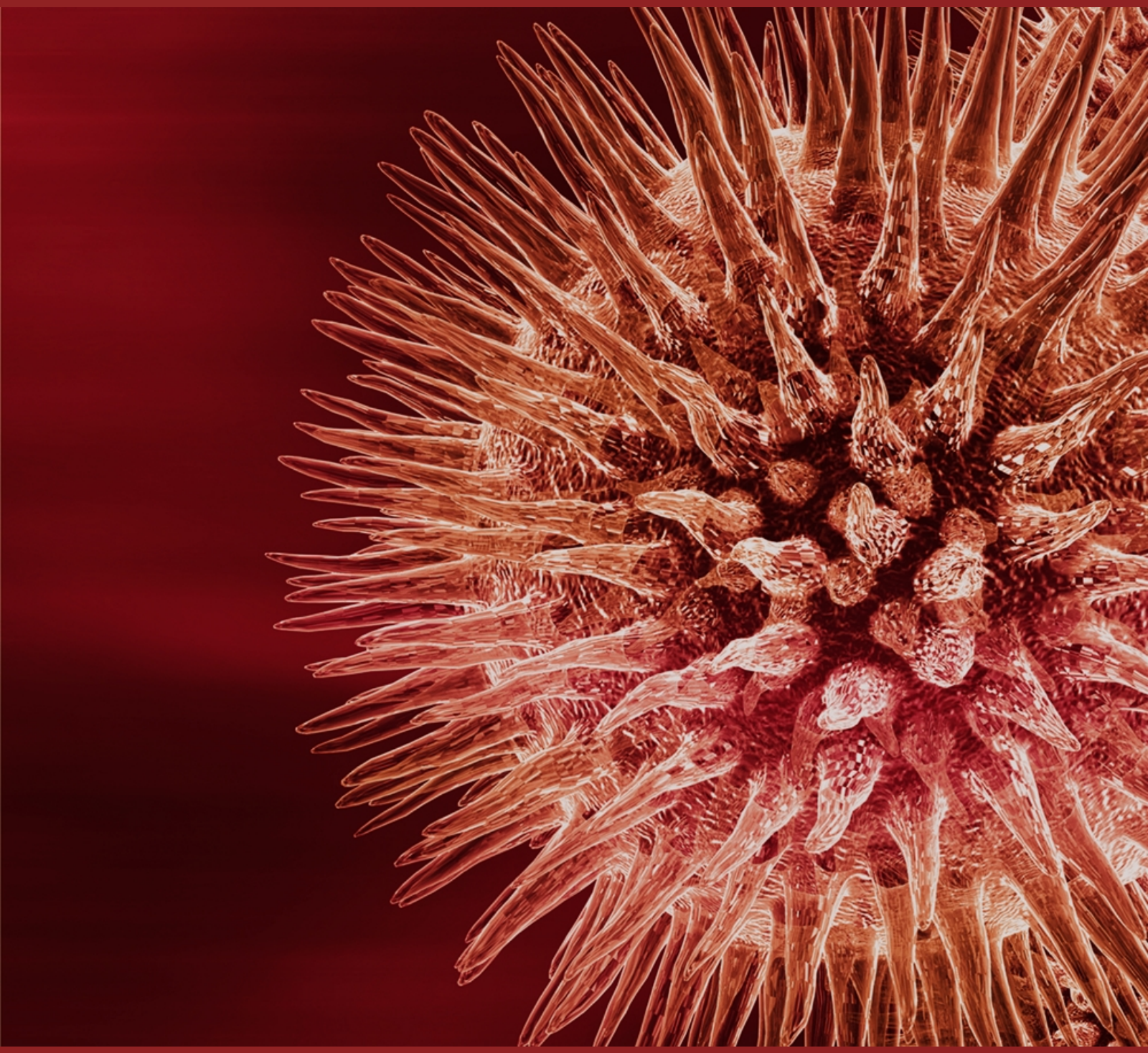
Journal of Biomedicine and Biotechnology

DNA Damage, Repair, and Diseases

Guest Editors: Lisa Wiesmüller, James M. Ford,
and Robert H. Schiestl



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Journal of Biomedicine and Biotechnology • 2:2 (2002) 45–45 • PII. S1110724302001985 • http://jbb.hindawi.com

EDITORIAL

DNA Damage, Repair, and Diseases

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DNA is the essential carrier of genetic information in all living cells. How is the huge amount of DNA in organisms from bacteria to humans maintained and protected from the ravages of noxious agents in the environment? The chemical stability of the DNA molecule is not unusually great, DNA undergoes several types of spontaneous modifications, and it can also react with many physical and chemical agents, of which some are endogenous products of the cellular metabolism (eg, reactive oxygen species) while others, including ionizing radiation and ultraviolet light, are threats from the external environment. The resulting alterations of DNA structure are generally incompatible with its essential role in preservation and transmission of genetic information. Damage to DNA can cause genetic alterations, and if genes that control cell growth are involved, these mutations can lead to the development of cancer. Of course, DNA damage may also result in cell death which can have serious consequences for the organism of which the cell is a part; for example, loss of irreplaceable neurons in the brain. Accumulation of damaged DNA has also been considered to contribute to some of the features of aging. It is not surprising that a complex set of cellular surveillance and repair mechanisms has evolved to reverse the potentially deleterious damage that would otherwise destroy the precious blueprint for life. Some of these DNA repair systems are so important that life cannot be sustained without them. An increasing number of human hereditary diseases that are characterized by severe developmental problems and/or a predisposition to cancer have been found to be linked to deficiencies in DNA repair.

The many types of DNA repair include excision repair mechanisms targeted to the removal of bulky DNA adducts and UV-induced photoproducts, base-pair alterations and purine loss, and DNA mismatches, and single- and double-strand DNA breaks. In addition, DNA replication, recombination and transcription are all involved in DNA repair pathways. A complex interplay between intrinsic hereditary factors and persisting DNA damage determines the susceptibility of humans to cancer. Inherited human diseases of DNA repair include many cancer susceptibility syndromes, such as Xeroderma pigmentosum, Ataxia-telangectasia, Bloom's and Werner's syndromes, Hereditary Non-Polyposis Colon Cancer, Li-Fraumeni-syndrome, and breast/ovarian cancer syndrome.

The most ubiquitous and versatile modes of DNA repair are those in which the damaged or incorrect part of a DNA strand is excised and then the resulting gap is filled by repair replication using the complementary strand as template. The excision repair pathway that deals with UV-irradiation induced pyrimidine dimers and a large variety of cancer-causing chemical adducts to DNA is known as nucleotide excision repair (NER). Several very interesting new developments in this field will be reviewed. These include DNA damage inducible responses in both prokaryotic and eukaryotic organisms, including both inducible DNA repair genes and genes involved in the proteasomal/ubiquitination pathway and the detection of such inducible responses using whole-genome transcriptional profiling techniques.

Recombination represents the irreplaceable repair mechanism under circumstances when DSBs appear as a result of metabolic processes or genotoxic treatment. In mammalian cells about half of all DSBs are repaired by homologous recombination, half by nonhomologous end joining. The lack of central enzyme functions can cause lethal phenotypes of knockout mice or result in extreme sensitivities towards ionizing radiation. Moreover, correct repair of DSBs is central to the maintenance of genomic integrity in mammalian cells, since errors give rise to translocations, deletions, duplications, expansions, and transposon integration, which accelerate the multistep process of tumor progression. Therefore, recombination processes are subject to surveillance by a hierarchy of genome stabilizing factors, such as p53 and the PI3 kinase ATM.

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Journal of Biomedicine and Biotechnology • 2:2 (2002) 46–54 • PII. S1110724302201011 • http://jbb.hindawi.com

REVIEW ARTICLE

Werner Syndrome

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Received 19 December 2001; accepted 4 January 2002

Werner syndrome is a premature aging disease caused by the mutation in the *WRN* gene. The cloning and characterization of the *WRN* gene and its product allows investigators to study the disease and the human aging process at molecular level. This review summarizes the recent progresses on various aspects of the *WRN* research including functional analysis of the protein, interactive cloning, complexes formation, mouse models, and SNPs (single nucleotide polymorphisms). These in depth investigations have greatly advanced our understanding of the disease and elucidated future research direction for Werner syndrome and the human aging process.

INTRODUCTION

Until recently, Werner syndrome (WS) had been considered a model of accelerated aging. The *WRN* gene was identified in 1996 [1], and was subsequently shown to act both as a DNA helicase and as an exonuclease [2, 3]. Since then, cancer researchers and those that study DNA metabolism have collaborated to further characterize WS and the function of the *WRN* gene. The more we understand about the *WRN* gene, the more we realize that WS is not merely involved in accelerated aging. WS certainly does not represent premature aging, in a sense that the characteristic aging phenotypes seen in WS are considerably different from those observed in normal elders. WS is now being more correctly recognized as a condition in which the lack of WRN protein (WRNp) results in an overall decline in the normal physiological functions of various organs, including those most frequently used to estimate the chronological age, such as skin and hair.

Since the identification of the *WRN* gene five years ago, various in vitro biochemical studies of WRNp have answered many of our initial questions regarding the helicase and exonuclease functions of this enzyme. Considerably more time will be required to answer the more difficult questions concerning the in vivo functions of *WRN* at the organism and cellular levels. The lack of mouse models of WS that mimic the human disorder currently limits our ability to carry out such studies. In this review, the known in vitro functions of WRNp and the cellular characteristics of WS cells will be summarized. From this, the putative in vivo functions of *WRN* will be extrapolated.

CLINICAL FEATURE OF WERNER SYNDROME

The clinical phenotypes of WS can be best summarized as onset of an aged-appearance and age-related common disorders [4, 5, 6, 7]. Unlike people with Hutchinson-Gilford progeria, WS patients usually develop normally until they reach the second decade of life. Some patients may present with flat feet. Generally, the first sign is a lack of the pubertal growth spurt during the teen years. Patients frequently recall that they were of average height when they entered grade school, but were the shortest ones in their class by the time they graduated from high school. In their 20s and 30s, patients begin to manifest skin atrophy, loss of hair, and graying hair. Subcutaneous fat tends to deposit on the trunk, and combined with osteoporosis of the limbs, patients exhibit a stocky appearance. The other most common age-related disorders seen in WS patients are bilateral cataracts and type II diabetes mellitus. Penetrances are 98% for cataracts and 90% for diabetes mellitus [7]. These numbers obviously depend on the age of the patient when clinical reports are made, and how rigorously patients were examined. Demographically, WS cases are most frequently reported in Japan. This can be partly attributed to an awareness of the syndrome among Japanese physicians, and the higher consanguinity among the Japanese population as compared to the US population. The chronological order of the onset of these complications is similar among Caucasian and Japanese WS patients [6, 7]. The International Registry of Werner Syndrome (Seattle, Wash, USA) has documented more than 100 cases of WS. New cases with a diagnosis confirmed by genetic analysis are mostly in patients over the age of 30, probably due to the lack of prominent symptoms prior to the age of 20.

Two common causes of death among WS patients are malignancy and myocardial infarction [6, 8]. Although these are also two common causes of death in the general population, unique characteristics are observed in WS patients. An extensive review of malignancies has been carried out in WS cases in Japan, where the prevalence of *WRN* mutations is relatively high [9]. Strikingly, the ratio between cancers of epithelial origin and sarcomas of mesenchymal origins is 1:1 in WS patients, whereas this ratio is approximately

10:1 in the general population. A possible explanation for the high representation of mesenchymal tumors in WS patients may be related to the mechanism by which telomere length is maintained [10]. Review of the pathological studies of these malignancies revealed unusual primary sites for cancers in WS patients. For example, melanomas in WS patients are of the acral lentigenous type in the mucosas, and are unrelated to sun exposure [9]. The primary sites of osteosarcoma in WS patients are more likely to be in the lower extremities, whereas these are more common in the upper extremities in the general population [11]. This variation may be related to the threshold of WRNp required to maintain DNA stability in each cell type within a given organ. Among WS patients, the specific cell type in which cancer develops may differ, depending on the type of mutation in the *WRN* gene. Papillary carcinoma has been associated with an N-terminal mutation, whereas follicular carcinoma is more frequently observed with a C-terminal mutation [5]. This finding clearly contradicts our original assumption that all identified mutations within *WRN* result in truncation of the nuclear localization signal of WRNp, and thereby act as null mutations (see Section "*WRN* mutations"). Further studies may reveal additional correlation between specific genotypes and phenotypes.

Several other important differences have been noted between WS patients and normal elders. Atherosclerosis exhibits unique characteristics in WS patients. Atherosclerotic lesions are more extensive in arterioles as compared to non-WS patients, who exhibit lesions primarily in major arteries. Calcification of cardiac valves is also sometimes observed in WS, possibly reflecting excessive cell death due to the constant pressure of the blood flow. Skin ulcers around the ankles and elbows that are more severe than those typically seen in the progression of diabetes mellitus are not uncommon in WS. Dementia of the Alzheimer type is relatively rare [12] despite the fact that WRNp is expressed in the brain [1]. Whereas in the general population, osteoporosis has a more pronounced effect on vertebrae, long bones, particularly those of the lower limbs, tend to be more affected by osteoporosis in WS patients [13].

Werner syndrome was first described by Dr Otto Werner at Kiel University in his doctoral thesis at the turn of the 20th century [14]. Two symptoms that drew his attention were bilateral cataracts and scleroderma-like skin. The latter turned out to provide insight into the interaction between WRNp and the Ku complex (see Section "Telomere maintenance"), the autoantigen that triggers scleroderma, an autoimmune disorder.

WRN GENE PRODUCT

Functional domains of WRN gene product

The *WRN* gene is comprised of 35 exons on the short arm of chromosome 8, and encodes protein of 1432 amino acids. It was identified in 1996 using a conventional positional cloning approach [1], and the methods that were available at that time [15]. Simple alignment in database searches and more complicated structural studies showed four defined regions of WRNp. These include exonuclease domains I, II, and III in the N-terminal region [16, 17]; RecQ-type helicase domains I, Ia, II, III, IV, V, and VI in the central region [18]; a RecQ conserved motif immediately following the helicase motifs [19]; and a helicase ribonuclease D C-terminal (HRDC) conserved motif in the C-terminal regions [19].

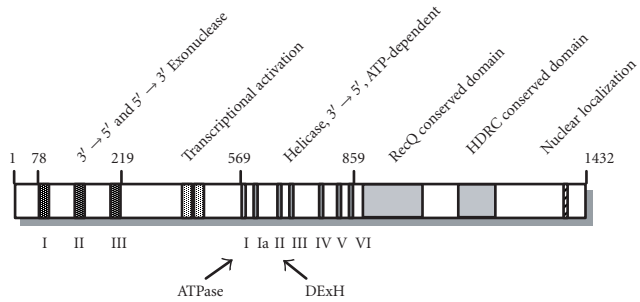

FIGURE 1. Functional domains of the WRN protein. Arabic numbers indicate amino acids of the WRN protein. The designated functions of the exonuclease domains (I–III), the acidic repeat transcriptional activator regions, helicase domains (I–VI), and nuclear localization signal have been demonstrated in various biological assays. The roles of the RecQ C-terminal conserved region and the helicase RNaseD C-terminal (HDRC) conserved regions are based on the structural studies.

Biochemical studies confirmed the helicase and exonuclease activity of WRNp in vitro [2, 3, 20]. The function of the RecQ conserved motif has not been defined. The HRDC motif is thought to form a scaffold which interacts with substrate DNA with relatively low affinity, based on the three-dimensional structure, which resembles the auxiliary DNA-binding domains of bacterial DNA helicases [21]. Cell biological studies identified two other functional domains. One of these functions as a transcriptional activator within a highly acidic region between the exonuclease and helicase domains [22, 23]. The other functions as a nuclear localization signal in the extreme C-terminus of WRNp [24, 25]. The relative locations of these structural domains are shown in Figure 1.

WRN mutations

To date, at least 35 different *WRN* mutations have been reported from all over the world [1, 8, 26, 27, 28, 29] (see Figure 2). The known mutations correspond to either stop codons, insertions, or deletions that result in a frame shift, or splicing donor or acceptor site mutations, which cause an exon to be skipped, resulting in a frame shift. All of these mutations result in truncation of the nuclear localization signal. Unlike the *BLM* gene [30], no missense mutation has been identified in WS. As we expand WS screening, this type of mutation may be identified in future WS cases.

In addition to the loss of the nuclear localization signal in WRN mutations, the mutant mRNAs, and the resulting mutant proteins exhibit shorter half-lives than do the wild-type mRNA, and WRNp [31, 32]. Mutant products truncated N-terminal to the helicase regions were more labile than a mutant protein truncated C-terminal to the helicase region.

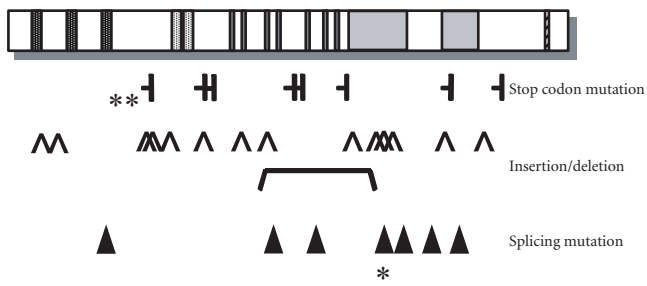

Figure 2. *WRN* mutations in WS patients. The rectangular boxes indicate the functional domains of the WRN protein (see Figure 1). Known *WRN* mutations are grouped based upon the type of mutation, shown above and underneath the WRN protein diagram along with Registry codes. Parentheses indicate the heterozygous mutations. R367Stp (∗∗) is the most common mutation seen in Caucasian WS patients (approximately 25%) and the second most common mutation in Japanese WS patients (approximately 18%) [33]. Deletion of exon 26 (∗), which is due to a splice junction mutation, is the most common mutation in Japanese WS patients (approximately 52%) [33].

The biological significance of low levels of mutant WRNp in the cytosol is unknown. Depending on the specific mutation, certain WRNp mutations can retain enzyme activity or other functions. These proteins can modify DNA during the mitotic phase or modify RNA during all phases of the cell cycles. It is possible that various mutant forms of WRNp, which may be retained in the cytosol, could contribute to the slight differences in phenotype observed with various WRN mutations. For example, thyroid cancer associated with the two major WRN mutations differs [5]. Papillary carcinoma is associated with the N-terminal mutation, while follicular carcinoma is seen more often with the C-terminal mutation.

WRN helicase activity—effect of single-strand binding proteins and substrate specificities

The helicase activity of WRNp is a RecQ-type helicase, named after the prototypic *Escherichia coli* RecQ. A number of laboratories have demonstrated that WRNp exhibits ATP-dependent $3' \rightarrow 5'$ helicase activity in an oligo-displacement assay [2, 20, 34, 35, 36, 37]. One study showed that this activity was present in an immunoprecipitated sample of WRNp [38]. WRNp appears to first bind to the single-stranded portion of the longer strand of the DNA duplex and proceeds in a $3' \rightarrow 5'$ direction with respect to the longer strand [35]. This $3' \rightarrow 5'$ movement corresponds to the direction of proofreading, as opposed to the direction of DNA synthesis, which proceeds from $5' \rightarrow 3'$. Thus, *WRN* has been proposed to play a role in some aspects of DNA strand repair, although the specific mechanisms involved are not known. WS cells are hypersensitive to 4-nitroquinoline-1-oxide (4NQO) [39], although WRNp does not have a higher binding affinity for DNA that has been damaged by 4NQO or by UV light [35]. The WRN helicase can also function to unwind DNA-RNA duplexes [20].

The helicase activity of WRNp can be enhanced by the presence of various single-strand binding proteins, such as *Escherichia coli* SSB, the T4 gene 32 product or, more efficiently, human replication protein A (hRPA) [2, 20, 35, 36, 37, 40]. These single-strand binding proteins are believed to facilitate *WRN* helicase activity by stabilizing single strand structures and by preventing their re-annealing. Several investigators have demonstrated that WRNp and hRPA are colocalized in *Xenopus* replication initiation complexes, and in Hela cells arrested in S-phase with hydroxyurea [41, 42, 43]. Moreover, recombinant WRNp and purified hRPA have been shown to co-immuno-precipitate [36]. hRPA might play an additional role, besides stabilizing the single-stranded DNA structure, in the *WRN*-catalyzed unwinding reaction, which requires direct interaction with WRNp.

Several unusual substrates have been tested as potential physiological targets for *WRN* helicase activity. *WRN* was able to efficiently unwind a G4 quartet made by two hairpin loops (G'2 biomolecular tetraplex) of d(CGG)n [44]. In this study, the G4 structure was generated under a high salt concentration in vitro. Although its presence has not been demonstrated in vivo, a G4 quartet can potentially be formed from two GC-rich regions of unwound single-stranded DNA (or RNA) during replication, repair, recombination, or transcription. Another interesting structure is a recombination intermediate, or α structure. WRNp was able to promote branch migration of a Holliday junction [42]. In fact, the *WRN* helicase appeared to dissociate the α structure better than a simple DNA duplex, as assessed by the length of the migration. This structure can be formed both during recombination and replication, such as break-induced DNA replication or repair of the stalled replication. The complex secondary structures described above were also substrates for the *BLM* helicase in vitro. Thus, in vivo, WRNp may participate in the dissociation of these structures as well.

Characteristics and significance of WRN exonuclease activity

The $3' \rightarrow 5'$ exonuclease activity of WRN has been demonstrated by at least four independent laboratories [3, 40, 45, 46, 47, 48, 49]. One group measured $5' \rightarrow 3'$ exonuclease activity [34], although no laboratory has demonstrated both $3' \rightarrow 5'$ and $5' \rightarrow 3'$ exonuclease activities in WRNp.

While exonuclease activity in the $3' \rightarrow 5'$ direction does not require adenine triphosphate (ATP), the enzyme activity is enhanced in the presence of ATP and is specific for the $3'$ recessed end of the duplex [3, 45, 46, 48]. The *WRN* exonuclease utilizes the $3'$ end of the blunt end as well as the $3'$ overhang end in presence of Ku70/80, the regulatory subunit of DNA protein kinase (DNA-PK) [47, 49]. Like its WRN helicase activity, the exonuclease activity of WRN also digests RNA-DNA heteroduplexes [48]. The exonuclease region of the mouse WRNp showed an activity [48].

Double-stranded DNA substrates with multiple base-pair mismatches within the duplex (termed *bubbles*) were more susceptible than those without mismatches to digestion by the *WRN* exonuclease, but the presence of more than two mismatches at the end of the substrate made them relatively more resistant to digestion [46, 48]. Structures resembling

Holliday junction are also known to be more susceptible to WRN exonuclease digestion than are simple DNA duplexes [46] similar to human WRNp.

Relative substrate specificity suggests that, like the *WRN* helicase, *WRN* exonuclease activity may be involved in repairing DNA damage. The exonuclease and helicase activities of *WRN* are physically and functionally separable [3]. How exonuclease and helicase functions are specifically coordinated during the DNA repair process remains unknown.

Regulation of transcriptional activation by WRN

Transcriptional activation of the WRN protein was originally suggested by findings in the yeast one-hybrid system [1]. Subsequently, transactivation of the adenovirus major late promoter was used as a reported plasmid to demonstrate that *WRN* can mediate transcription activation [23]. This deficit was reversed by the addition of recombinant WRN protein. Interestingly, a mutant WRN protein defective in helicase activity (K577M) was unable to complement the reduced transactivation in WS nuclear lysates, indicating that the transactivation function of *WRN* is coupled with its helicase/ATPase activities [23].

WRN PROTEIN COMPLEXES AND CELLULAR FUNCTION OF *WRN*

Identification of proteins that interact with *WRN* has helped to shed light on the in vivo functions of WRNp. Some laboratories have characterized the association of *WRN* with specific candidate interacting proteins, while others have screened cDNA libraries by the yeast two-hybrid system, isolated the *WRN* complex from the cell extracts, or captured the interacting proteins by binding to a WRNp affinity column. Interestingly, each method identified different sets of WRN interacting proteins. Thus, *WRN* may associate with different protein complexes, depending on the status of DNA metabolism during the time that the WRN protein was recruited.

Cell biological studies also suggested that *WRN* has multiple functions. Two characteristics of WS cells that had been well known, even before the *WRN* gene was identified, were that these cells exhibit a shortened replicative lifespan and genomic instability. More recently, studies of drug sensitivities and telomere metabolism led us to a better understanding of the specific functions of WRNp. Interacting proteins combined with cellular and subnuclear studies suggests that *WRN* may be involved in a wide variety of DNA metabolic processes.

DNA replication

FFA-1 is a *Xenopus* ortholog of *WRN*, and was originally identified in the replication initiation complex of *Xenopus* oocytes as being required for the formation of replication foci during DNA replication [41]. FFA-1 and the p70 subunit of RPA have also been co-immunoprecipitated from lysates of *Xenopus* oocytes [43]. WRNp interacts with several components of the DNA replication complex, including

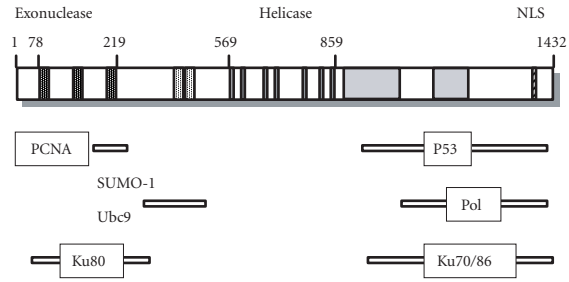

FIGURE 3. Proteins that interact with the WRN protein. Proteins known to interact with WRNp at specific sites are shown schematically. The approximate regions of protein-protein interaction are indicated.

proliferating cell nuclear antigen (PCNA) and topoisomerase I [50]. The N-terminal region within the exonuclease domain of the WRNp, particularly the region including amino acids 168–246, strongly interacted with the C-terminal portion of PCNA [50], as indicated in Figure 3. The N-terminal region of recombinant WRNp appears to form mostly trimers, as does PCNA, suggesting that the interaction between *WRN* and PCNA involves quaternary structure [48].

DNA polymerase δ

DNA polymerase δ (pol δ) participates in DNA replication and repair of DNA damage. This enzyme is also found in the telomere complex. Evidence of physical interaction between *WRN* and pol δ initially came from a yeast two-hybrid screen, in which the C-terminal region of WRNp was used as the "bait" to capture the p50 subunit of pol δ, as shown in Figure 3 [51]. Immunoprecipitation with an anti-p50 antibody resulted in a complex including p120, the catalytic subunit of pol δ. In yeast, physical, and functional interaction between *WRN*, and pol δ requires the third subunit pol32p [52].

Functional interaction of WRNp, and pol δ appears to be unilateral. In a simple primer extension assay, the addition of WRNp enhanced pol δ activity, whereas the addition of pol δ did not stimulate exonulcease or helicase activities [52]. In contrast, WRNp did not stimulate either pol α- or pol ε-mediated DNA synthesis [52, 53]. The presence of WRNp also enabled pol δ to transverse hairpin and G'2 bimolecular tetraplex structures to complete DNA synthesis [53]. One of the functions of WRNp may be to recruit pol δ to the complex secondary DNA structure and to alleviate stalled DNA synthesis [51, 53].

Homologous recombination

One of the important biological functions of *E coli* RecQ is to suppress homologous recombination by disrupting its intermediate structure [54]. The yeast homolog of RecQ, Sgs1, is involved in both homologous recombination and in illegitimate recombination or nonhomologous end joining (NHEJ). Both the *WRN* and *BLM* helicases are able to complement increased homologous recombination and illegitimate recombination of an Sgs1 deletion

mutant [55]. However, the cytogenetic characterization of cells from Bloom patients shows increased exchanges of sister chromatids, whereas cells from WS patients exhibit translocation mosaicism [56]. These findings suggest that the *BLM* helicase may play a more prominent role in the suppression of homologous recombination in vivo.

Repair of breaks in double-stranded DNA

DNA-PK is a protein complex including a protein kinase catalytic subunit, DNA-PKcs, and a regulatory subunit, Ku70/80 [57]. This protein complex is involved in the initial stages of NHEJ. WRNp has been shown to directly interact with DNA-PKcs and Ku80 [47, 49, 57]. Association of *WRN* with the Ku complex enhances its exonuclease activity, but has no effect on its helicase activity [49]. Upon binding to Ku, *WRN* is able to degrade DNA from both the 5′ recessed end (5′ → 3′ exonuclese activity), and from the blunt end. This may explain why both 3′ → 5′ [3, 45, 58], and 5′ → 3′ [34] exonuclease activities were observed in recombinant WRNp. The presence of exonuclease activity has been speculated to be a necessary step for DNA-PK mediated NHEJ prior to the polymerization, and ligation step mediated by XRCC 4 and ligase IV [57]. In addition, the *WRN* helicase may unwind broken ends of dsDNA in the search for microhomology.

Telomere maintenance

In a high quality immunofluorescence study, Shiratori et al [59] identified nuclear dots suggesting that WRNp is localized on telomeres. Johnson et al [10] demonstrated WRNp co-localizes with various telomere components, including TRFI and TRFII in six ALT cell lines. ALT cells lack telomerase activity, and the telomeres in these cells are presumably maintained by recombination. Recombinant WRNp did not unwind a G'2 tetraplex containing a telomere repeat sequence [44]. However, WRNp did appear to unwind up to 23 kb of a PCR-generated telomere repeat sequence to ssDNA, and this process was stabilized by hRPA [60]. These findings collectively suggest that WRNp plays an important role in telomere maintenance.

During the serial passage of primary WS fibroblasts, telomeres became shortened more quickly, and telomeres in cells that had stopped dividing were longer than those in control fibroblasts [61]. Telomeres of WS Lymphoblastoid cell lines (LCLs) were unstable, and telomere length varied more widely in LCLs from WS patients as compared to those from normal subjects [62]. The telomere length at which LCLs from WS patients went into crisis also varied widely. It has been suggested that one way in which WRNp may be involved in the ALT pathway is by regulating the number of extra-chromosomal telomere repeats [63]. This may explain why the catalytic subunit of human telomerase (hTERT) is able to extend the replicative lifespan of WS cells indefinitely [64, 65, 66]. hTERT may be able to circumvent the early halt of cell division in WS cells because the WRNp complex functions in pathways that are distinct from hTERT-dependent telomere maintenance [63]. These findings provided significant insight into the possible mechanism of mesenchymal tumorigenesis in WS patients, as ALT cell lines are frequently mensenchymal in origin [10].

However, other important factors may also be involved in regulation of telomere function. Hisama et al [67] showed that both *WRN* and hTERT could complement 4NQO sensitivity in SV40 transformed WS fibroblasts. Introduction of hTERT appeared to reprogram gene expression [66]. WRNp may play an additional role in telomere maintenance, aside from repair of damaged telomeres and immortalizing cells that lack telomerase activity.

p53

As indicated in Figure 3, the C-terminal region of WRNp interacts with the p53 tumor suppressor, as these proteins can be co-immunoprecipitated [68, 69]. In the absence of *WRN*, p53-mediated apoptosis is attenuated [68]. Overexpression of *WRN* enhances p53-dependent transcriptional activation of p21Waf1 [69], and potentiates p53-mediated apoptosis [69]. Synergistic actions of p53 and *WRN* have been observed in mouse models of WS [70, 71].

SUMO-1

A yeast two-hybrid screen using mouse *WRN* as the bait identified Ubc9 and SUMO-1 as *WRN* interacting proteins [72]. The N-terminal domain (amino acids 272–514) of *WRN* interacts with both Ubc9 and SUMO-1.

MOUSE MODELS OF WERNER SYNDROME

There are currently three mouse models of Werner syndrome:

- *WRN* helicase domain deletion mice (WRNΔhel/Δhel) [73],
- WRN truncation mutant mice (presumably null, WRN−/−) [70], and
- transgenic mice expressing dominant negative WRN (K577M-WRN) [74].

These various genetic mutations are depicted schematically in Figure 4.

WRN deficient mice carry a *WRN* gene with a frame deletion of the helicase domains III and IV, as described by Lebel and Leder [73]. These mice express a mutant *WRN*, WRNΔhel/Δhel, which retains the exonuclease domains and nuclear localization signal. Mouse ES cells showed hypersensitivity to campthothecin, and mitomycin C and the growth rate of embryonic fibroblasts was progressively reduced as the cells were passaged. The mice exhibited a normal phenotype at least until the age of 12 months [71].

Another mouse line expresses a mutant WRN protein that is truncated in the middle of the helicase region, resulting in mice that are functionally null (WRN−/−) [70]. This mutation resembles many of the mutations in WS

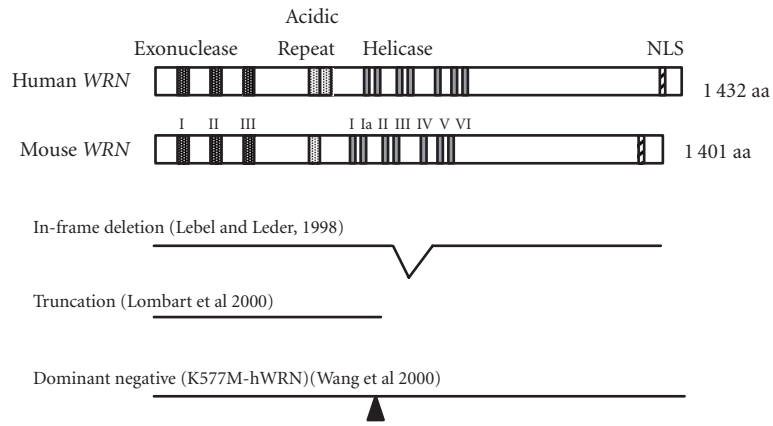

Figure 4. Mouse models of Werner syndrome. Structures of human and mouse WRN proteins and the alterations in the *WRN* gene product in the three mouse models of WS are shown.

patients. Embryonic fibroblasts showed accelerated replicative senescence, and this was enhanced in cells from mice on a BLM+/– background. They did not show hypersensitivity to campthothecin or 4NQO. Histopathological studies failed to show any unusual lesions in these mice up to the age of 17 months [70].

Mice with either a deletion or a truncation in the *WRN* helicase domain showed gross abnormalities when they were crossed to mice on a p53 – /– background. WRN+/– mice showed increased rates of mortality, as assessed by both median survival and maximum survival of the cohort of 48 WRN–/–; p53 – /– and 59 WRN+/–; p53 – /– mice [70]. Median lifespan of WRN–/–; p53 – /– mice was approximately 20% shorter than that of WRN+/–; p53 – /– mice. Interestingly, WRNΔhel/Δhel; p53 – /– mice exhibited increased numbers of tumors, and a larger variety of tumor types at an earlier age, as compared to WRN+/+; p53 – /– mice [71]. Both studies suggest that *WRN* and p53 play a synergistic role in the maintenance of genomic stability.

The dominant negative *WRN* transgenic mouse line, K577M-*WRN*, was developed in my laboratory [74]. These mice express both a full length mutant (K577M) human WRN and endogenous mouse *WRN*. The K577M mutation abolishes the helicase and ATP-ase activity of *WRN* in vitro, but not the exonuclease activity [2, 3]. Tail fibroblasts from K577M-WRN mice showed hypersensitivity to 4NQO and a reduced replicative lifespan, as determined by clone size distribution [74]. These mice did not show any histopathological abnormalities at least up to the age of 18 months.

To date, there is no animal model of WS that accurately mimics the human disease. This is also true for some, if not all, other mouse models of RecQ helicase-deficient disorders. This lack of success in reproducing human disease states in rodents may be due to the difference between humans and mice in maintaining genomic instability. Nevertheless, these mouse models have greatly facilitated the characterization of the functional interaction of WRNp with other proteins and of the pathogenesis of WS phenotypes.

WRN POLYMERASE AND ITS ROLE OF *WRN* IN "NORMAL" AGING

A systematic search of the *WRN* polymorphism across a variety of ethnic groups identified 58 SNPs, single nucleotide polymorphisms, in 35 exons, and flanking introns. Of these, 15 are localized within the coding region and 11 of these result in alterations in amino acid sequence [75, 76]. A limited association study of the two known *WRN* SNPs with relatively high heterogeneity suggested that *WRN* may be involved in determining longevity, possibly by modulating the risk of a variety of common age-related disorders, including atherosclerosis. Statistically significant associations with longevity and atherosclerosis were observed for the 1074Leu/Phe mutation [77]. In addition, the 1367Cys/Arg mutation was associated with myocardial infarction [78], atherosclerosis [77, 79], and long-term hemodialysis [80]. These two polymorphic sites are in linkage. The functional manifestations of these two SNPs, with regard to *WRN* function, are unknown. However, it is of interest to note that 1074Leu/Phe resides within the RecQ consensus domain and 1367Cys/Arg is four amino acids away from the nuclear localization signal. Interestingly, in 1367Cys/Arg, the beneficial allele, Arg, is a minor allele. This might be explained by the presence of linkage disequilibrium between 1074 Leu/Phe and 1367 Cys/Arg in some populations [76, 77]. The relative contribution of WRN polymorphisms to the risk of age-related disorders or longevity may not be nearly as high as other major known genetic risk factors, for example, polymorphisms in ApoE [75]. However, these studies are beginning to provide hints that *WRN* may indeed be involved in the "normal" aging process in the general population.

SUMMARY

Following the initial biochemical characterization of the helicase and exonuclease activities of WRNp, the identification of *WRN*-interacting proteins has led to a variety of studies exploring various ways in which WRNp may be involved

in DNA metabolism. These include DNA repair at the site of stalled replication and NHEJ. *WRN* may also have a special role in transcription by RNA polymerases I and II, as well as in homologous recombination. Cell biological studies raise an interesting question regarding the role of *WRN* in telomere maintenance. If *WRN* is capable of all these functions, how do cells regulate the functions of *WRN* at any given time? How is the switching of these roles mediated? How are the relationships with other RecQ helicases regulated? Since symptoms of WS are relatively mild (not lethal), *WRN* may have evolved for the "fine tuning" of these various DNA metabolisms. If this hypothesis is true, what are the driving forces for the origin of *WRN*? At the animal level, how is *WRN* involved in the progression of "normal" aging phenotypes? I look forward to learning the answers to these and other important questions during the coming decades.

ACKNOWLEDGMENT

The author thanks Mr Kevin Fung and Mr Nhut Vuong for the editorial assistance.

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Journal of Biomedicine and Biotechnology • 2:2 (2002) 55–60 • PII. S1110724302201023 • http://jbb.hindawi.com

REVIEW ARTICLE

Nucleotide Excision Repair, Genome Stability, and Human Disease: New Insight from Model Systems

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Received 18 December 2001; accepted 4 January 2002

Nucleotide excision repair (NER) is one of several DNA repair pathways that are universal throughout phylogeny. NER has a broad substrate specificity and is capable of removing several classes of lesions to the DNA, including those that accumulate upon exposure to UV radiation. The loss of this activity in NER-defective mutants gives rise to characteristic sensitivities to UV that, in humans, is manifested as a greatly elevated sensitivity to exposure to the sun. Xeroderma pigmentosum (XP), Cockaynes syndrome (CS), and trichothiodystrophy (TTD) are three, rare, recessively inherited human diseases that are linked to these defects. Interestingly, some of the symptoms in afflicted individuals appear to be due to defects in transcription, the result of the dual functionality of several components of the NER apparatus as parts of transcription factor IIH (TFIIH). Studies with several model systems have revealed that the genetic and biochemical features of NER are extraordinarily conserved in eukaryotes. One system that has been studied very closely is the budding yeast *Saccharomyces cerevisiae*. While many yeast NER mutants display the expected increases in UV sensitivity and defective transcription, other interesting phenotypes have also been observed. Elevated mutation and recombination rates, as well as increased frequencies of genome rearrangement by retrotransposon movement and recombination between short genomic sequences have been documented. The potential relevance of these novel phenotypes to disease in humans is discussed.

DNA REPAIR AND DISEASE

The maintenance of the integrity of DNA is of a paramount biological importance as it serves as the central repository for all genetic information. A network of biochemical pathways exists in all cells to maintain the informational and structural fidelity of DNA. These pathways are characterized, in part, by the types of modifications to the DNA to which they respond. The dissection of these pathways has involved a coordinated biochemical, genetic, and molecular biological approach where specific enzymatic defects are correlated with mutations at particular chromosomal loci, and distinctive cellular phenotypes. In humans, the specific DNA repair defects have been correlated with particular inherited diseases. Hereditary nonpolyposis colorectal cancer, for example, is a disorder associated with mutations in genes encoding components of the mismatch repair apparatus that lead to profound defects in cellular mismatch repair, and increases in genome instability [1]. Similarly, a trio of phenotypically disparate diseases, XP, CS, and TTD have been connected with mutations in genes that encode subunits of the NER and TFIIH apparatus, that confer measurable defects in NER and transcription [2, 3, 4]. The broad symptomology of these diseases most likely reflects the multiple biochemical defects caused by the mutations, some of which have not been extensively explored. This work focus on some of the less described effects of mutations in the NER/TFIIH apparatus on genome stability, and their potential implications with respect to human disease.

NUCLEOTIDE EXCISION REPAIR

NER plays a critical role in the maintenance of genomic integrity because of its broad substrate specifity. It plays an important role in the removal of such diverse lesions as UV light induced photoproducts as well as chemically induced bulky adducts, crosslinks, and oxidized bases. NER has been functionally conserved throughout phylogeny, although the apparatus in prokaryotes and eukaryotes is substantially different. NER in eukaryotes involves the concerted action of up to 30 proteins and has been reconstituted in vitro [5, 6]. NER occurs in the context of two distinct pathways related to the way the lesions are identified. General genome repair (GGR) responds to genome-wide damage, which in mammals is recognized by the XPC/HHR23B complex [7]. Transcription coupled repair (TCR) is focused exclusively on the repair of lesions on the transcribed strand of actively transcribed genes [8], and is thought to involve recognition of RNA polymerase II stalled at a lesion by the CSA and CSB proteins [9, 10]. The *XPA* protein then orchestrates the assembly of a complex

including the single-stranded DNA binding protein RPA and the core of the TFIIH complex at the site of the lesion [11]. This complex unwinds the DNA surrounding the lesion in an ATP-dependent process that involves the helicase activities of the *XPB* and *XPD* proteins, components of the TFIIH core complex [12, 13]. Interestingly, only the helicase activity of *XPB* is required for TFIIH-mediated melting of promoter DNA during the initiation of transcription, while *XPD* helicase activity plays a minor role in RNA polymerase II promoter escape [14]. Two structure-specific endonucleases, the ERCC1/XPF heterodimer, that cleaves on the 3′ side of the lesion, and *XPG*, that cleaves on the 5′ side of the lesion, are responsible for the removal of a 24–32 base oligonucleotide containing the lesion [15, 16]. The resulting gap is filled by DNA polymerases δ and ε, and repair is completed by ligation [5].

NER PHOTOSENSITIVITY SYNDROMES

XP, CS, and TTD have been ascribed to changes in NER and TFIIH function. Complementation analysis, conducted by analyzing the phenotypes of cells derived from the fusion of cells from different patients, identified several complementation groups for each disease [11]. Seven complementation groups, designated XP-A through XP-G, have been identified for XP. Two complementation groups, CS-A and CS-B, have been identified for CS. Three complementation groups, XP-B, XP-D, and XP-G, have been determined for the combined CS/XP patients. Three complementation groups, XP-B, XP-D, and TTD-A, have been identified for TTD. Most of the genes corresponding to these complementation groups have been cloned and the mutations responsible for disease in individual kindreds have been identified.

XP, CS, and TTD patients have distinct symptoms that likely reflect the participation of the mutant proteins in multiple biochemical processes. In patients, symptoms can often be correlated with specific biochemical defects. An example for each disease is listed below. XP patients exhibit distinct alterations of the texture and pigmentation of the skin that are related to exposure to the sun. They are also enormously predisposed to both sunlight-induced and internal cancers, consistent with defects in NER [17]. For example, an 80% reduction in cellular NER activity in an XP patient homozygous for a particular *XPF* mutation correlates well with the acute reactions to sun exposure and skin cancer that were also observed [18]. CS patients present symptoms of poor growth and developmental deformity that are most likely due to defective transcription. Accordingly, extracts of cells from patients with particular *CSA* and *CSB* mutations have been shown to support reduced levels of RNA polymerase II mediated transcription, as have cells from patients with combined XP/CS disease that are due to a mutation in *XPB* [19]. TTD patients suffer from brittle hair and nails, scaly skin and developmental abnormalities that are probably related to defects in NER and transcription. In support of this, fever inducible hair loss was correlated with temperature sensitive transcription and NER in the cells of patients with specific *XPD* mutations [20].

STUDIES WITH MODEL SYSTEMS

The extraordinary level of conservation of the NER and TFIIH apparatus among eukaryotes permits the extensive use of model systems to better explore the genetic and biochemical control of theses processes. Studies in rodents, flies, plants, and fungi have all contributed to the understanding of the role played by the NER and TFIIH apparatus in eukaryotes [21, 22, 23, 24, 25, 26]. However, trangenic mice and budding yeast have been particularly helpful in studying NER and TFIIH at the molecular level, as well as their effects on the phenotype of the organism. For example, in a recent study, isogenic strains of mice homozygous for null alleles of *XPA* or *CSB* were found to be extremely sensitive to a carcinogen that forms bulky adducts on DNA, while an *XPC* null strain was not [27]. This suggests that *XPA*-dependent and CSB-dependent TCR is critical for survival to exposure to an important class of carcinogens, while *XPC*-dependent GGR is not. Such studies are very important in determining how mammals respond to particular types of DNA damage. Mice have also been used to further explore the phenotypic effects of NER mutations known to cause disease in humans. For example, a strain of mouse was created that was homozygous for an *XPD* mutation analogous to the one found in several NER-deficient TTD patients [28]. The mice exhibited hypersensitivity to UV exposure and a cellular NER defect that was very similar to the TTD symptomology but, also exhibited a marked propensity toward UV-induced and carcinogen-induced skin cancer that was not observed in patients. This suggests the possibility that the NER defects observed in TTD patients could lead to cancer, an observation of potential clinical importance.

The study of NER and TFIIH in budding yeast and mammalian cells has been essentially contemporaneous. The observation that NER and transcription are genetically and biochemically linked occurred nearly simultaneously in the two systems, significantly accelerating the description of eukaryotic NER and transcription initiation at the molecular level [29, 30]. The degree of similarity between the apparatus in humans and yeast is extraordinary, permitting investigators to examine the impact of disease causing alleles of human NER genes on yeast. In one study, two TTD alleles of *XPD* were unable to complement the lethal effect of a null allele of the *RAD3* gene, the budding yeast homolog of *XPD*, while wild-type and helicase-defective alleles of *XPD* were able to complement [31]. Since the essential function of Rad3/XPD is thought to be its role in transcription initiation, the authors concluded that the TTD alleles confer a transcription defect, and, therefore, that the disease may be due to defective transcription of a critical gene, or genes.

NER, TFIIH, AND THE MAINTENANCE OF GENOME STABILITY IN BUDDING YEAST

The careful study of NER in budding yeast has revealed its impact on cellular processes yet to be recognized in other organisms. These unexpected relationships could provide

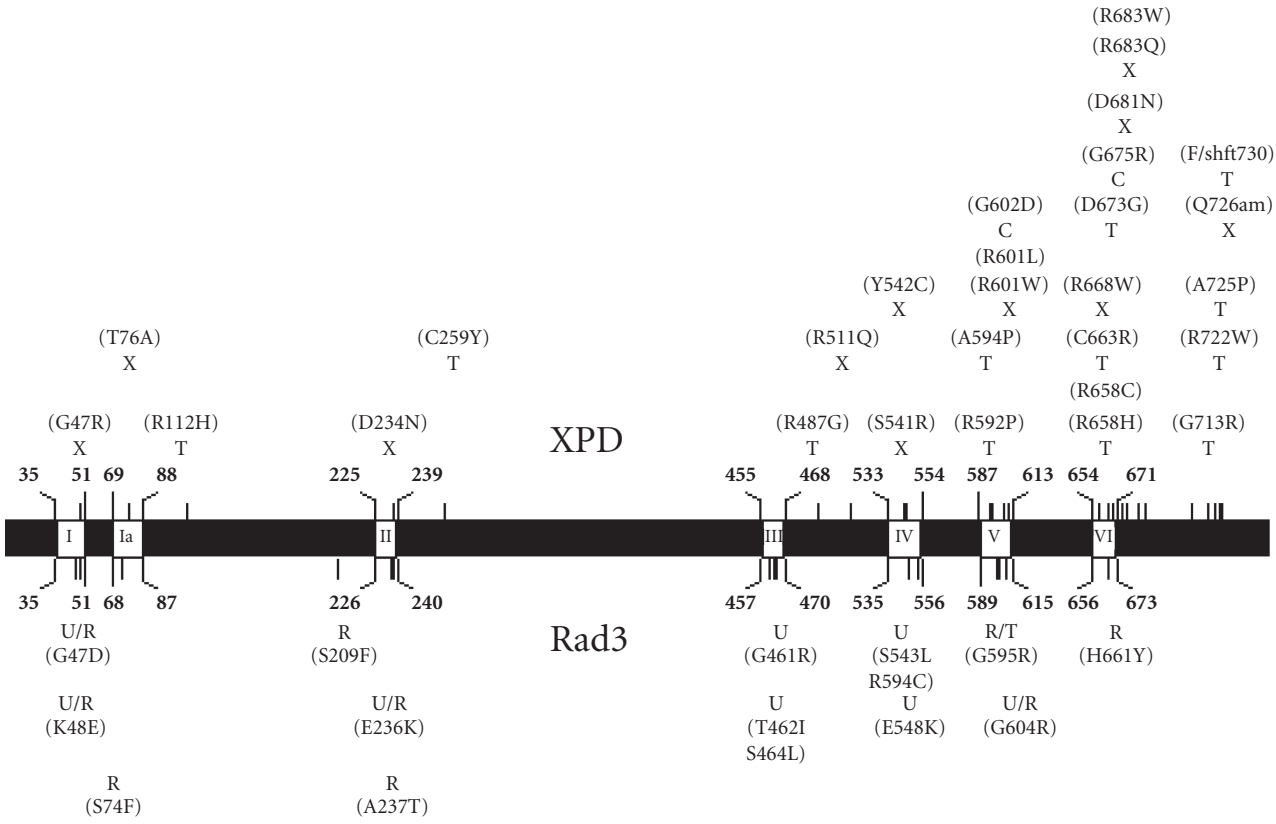

Figure 1. Amino acid changes in the human XPD and budding yeast Rad3 helicases that lead to phenotypic changes. The primary amino acid sequences of human XPD and yeast Rad3 are represented by the black bar. The seven conserved helicase domains are represented by white boxes and are named according to the accepted nomenclature [42]. The positions of these domains in the primary sequence are listed in bold print as described in [46]. Positions of altered residues in the primary sequence of XPD in XP, XP/CS, and TTD patients [44] are marked with a light hash mark above the black bar. The corresponding diseases are marked with an X for XP, a C for XP/CS, and a T for TTD. The amino acid changes are listed in parentheses. Changes in the Rad3 primary sequence [38, 39] are represented by hash marks below the black bar. The corresponding phenotypic changes are denoted by a U for UV sensitive, an R for elevated recombination, and/or mutation, and a T for transcription defective. Corresponding amino acid changes are listed in parentheses.

insight into clinically important roles for the human NER apparatus that have yet to be carefully explored, especially when the disease phenotype is complex as in CS and TTD. An important example is provided by the *RAD1* and *RAD10* genes, homologs of *XPF* and *ERCC1*. Null mutations in *RAD1* and *RAD10* confer profound defects in several different types of homologous recombination [32, 33]. Studies analyzing the recombinational repair of defined double-strand breaks (DSBs) revealed that *RAD1* and *RAD10* were required to remove nonhomologous sequences from the ends of recombining molecules, an important step in the creation of certain genome rearrangments [34]. *RAD1* and *RAD10* also play a critical role in recombination between short sequences, which is likely to be vital for the maintenance of genome stability [35]. All of these effects are apt to be related to the role of the Rad1-Rad10 nuclease in the cleavage of important recombination intermediates [34, 35, 36].

The TFIIH machinery has also been implicated in the maintenance of genome stability. Mutant alleles of *RAD3*, *SSL1*, and *SSL2* (homologs of *XPD*, *P44*, and *XPB*) have been isolated that confer elevated rates of mutation and recombination. Several *rad3* mutants have been isolated that exhibit increased rates of mutation and/or recombination but not defective transcription nor NER, indicating that Rad3 possesses important cellular activities that are independent of transcription and NER [37, 38]. Interestingly, a pair of these mutant alleles, *rad3-101* (S74F) and *rad3-102* (H661Y), accumulate DSBs and are synthetically lethal in combination with mutations in recombinational repair genes, strongly suggesting that Rad3 influences either the creation of DSBs, or their processing by homologous recombination [38]. Another allele of *RAD3*, *rad3-G595R*, blocks the degradation of DSBs, which stimulates genome rearrangement by recombination between short repeated sequences [35, 36, 37, 38, 39, 40, 41]. The analogous mutations in *XPD* are of potential medical importance as they would be within regions that encode conserved helicase domains of the protein that are mutated in XP, TTD, and XP/CS patients (Figure 1), [42, 43, 44]. Since specific mutations in *SSL1* and *SSL2* that encode additional members of the heteromeric core of budding yeast TFIIH [29] also stimulate short-sequence recombination (SSR) by

blocking the degradation of DSBs [40, 41], it appears that TFIIH acts to maintain genome stability by restricting recombination between repetitive DNA sequences. We speculate that SSR could potentially contribute to the symptoms observed in XP, XP-CS, and TTD patients, as SSR in the human genome has been found to lead to a variety of diseases [45].

Recently, it was revealed that by restricting the movement of the retrotransposon Ty1, TFIIH plays an additional, critical role in the maintenance of genome stability in budding yeast. Ty1 can move either by integrase mediated transposition, or by insertion of Ty cDNA into existing Ty or δ elements (the Ty1 long-terminal repeat) by homologous recombination [47]. Components of TFIIH were discovered to play a role in restricting Ty mobility when specific *rad3* and *ssl2* mutants with increased levels of Ty1 movement were isolated [48]. Other *rad3* and *ssl2* mutants that confer extreme UV sensitivity do not stimulate Ty mobility, separating the NER and Ty maintenance functions of these genes. The increases in Ty mobility were correlated with a substantial elevation of the steady-state level of Ty1 cDNA, suggesting either that the *rad3-RTT* (regulator of Ty transposition) and *ssl2-RTT* mutations increase the synthesis of Ty1 cDNA, or decrease its degradation. Importantly, the level of Ty1 RNA and Ty1 proteins remained unchanged in the *rad3* and *ssl2* mutants. Another interesting observation is that other NER genes, such as *RAD2* and *RAD1* do not appear to be involved in Ty transposition [49].

In a subsequent study, the increased level of Ty1 cDNA was found not to be due to increased cDNA synthesis suggesting that TFIIH plays a role in the degradation of Ty1 cDNA [41]. This same study linked TFIIH-control of SSR and Ty1 movement by showing that *rad3-G595R* and *ssl2-RTT* increase both SSR and Ty1 movement while increasing the stability of both Ty1 cDNA and DSBs. It remains unclear whether TFIIH plays a direct or an indirect role in the degradation of the ends of DNA molecules, however, the helicase activities of Rad3 and Ssl2 suggest that TFIIH could play a role in opening the ends of DNA molecules, thereby facilitating their processing by exonucleases.

Blocking the degradation of DSBs and Ty1 cDNAs could enhance Ty1 movement and SSR in multiple ways. It could preserve sequences at the ends of the molecules that are important for SSR and Ty1 movement. For example, a DSB in a short repetitive sequence creates ends that are never far from the border of homology with potential donor repeats, such that any erosion of the ends would significantly decrease their ability to pair and recombine. Similarly, loss of the sequences at the ends of Ty1 cDNA would block transposition by Ty1 integrase. Another way (that increasing the stability of DNA ends might elevate SSR and Ty mobility) could be by prolonging the signal that elicits the DNA damage checkpoint. Pausing the cell cycle for a protracted period may improve the likelihood that the ends of DSBs in short repeats can find homologous sequences, or that the Ty1 preintegration complex can complete a transposition event. Changes in checkpoint activity have been shown to affect the frequencies of other genome rearrangements [50]. These and other mechanisms could be working simultaneously to increase genome rearrangement in *rad3* and *ssl2* mutant budding yeast. By extension, similar defects in the cells of XP, XP-CS, or TTD patients with mutations in the *XPD* or *XPB* genes might contribute to these diseases.

ACKNOWLEDGMENTS

This research was sponsored in part by the National Cancer Institute, Department of Health and Human Services. A. M. Bailis is funded by Public Health Service Grant GM57484.

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Journal of Biomedicine and Biotechnology • 2:2 (2002) 61–65 • PII. S1110724302201035 • http://jbb.hindawi.com

REVIEW ARTICLE

Ku and the Stability of the Genome

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Received 1 November 2001; revised 26 November 2001; accepted 11 December 2001

Ku proteins are associated with a variety of cellular processes such as repair of DNA-double-strand breaks, telomere maintenance and retrotransposition. In recent years, we have learned a lot about their cellular and molecular functions and it has turned out that Ku-dependent processes affect the stability of the genome, both positively and negatively, in several ways. This article gives an overview on the role of Ku in determining the shape of the genome.

INTRODUCTION

Ku proteins are heterodimers consisting of subunits with sizes of about 70 kd and about 80 kd, respectively, that are conserved in a variety of eukaryotes [1]. The heterodimer binds with high affinity to double-stranded DNA ends and hairpin structures. In mammalian cells, Ku bound to DNA ends activates the catalytic subunit of the DNA-dependent protein kinase (DNA-PK) and phenotypes of Ku-deficient cells largely overlap with phenotypes of cells deficient in this catalytic subunit. The yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* lack the catalytic subunit of DNA-PK. Nevertheless, the functions of Ku seem to be very similar in these lower eukaryotes and in mammalian cells. Involvement of Ku in the repair of DNA double-strand breaks (DSB) was not only demonstrated in yeast and mammalian cells, but also in *Drosophila melanogaster*, *Xenopus laevis*, and chicken [2, 3, 4]. Ku participates also in V(D)J recombination and immunoglobulin gene class switch [5, 6, 7], where joining between distant genomic regions is initiated by site- or region-specific DSB, respectively. At least in yeast and mammalian cells, Ku has an additional function in telomere maintenance, whose absence causes genomic rearrangements, and in processes associated with retrotransposition.

KU AND THE FIDELITY OF DSB REPAIR

All known pathways for the elimination of DSB can lead to genetic alterations. Homologous recombination can result in sequence alterations due to gene conversion mechanisms if the donor and the receptor molecule differ within the repair patch region (see [8] for a review of homologous recombination).

When associated with a cross-over, interchromosomal homologous recombination can also lead to a recombination of markers flanking the repair patch. In the case of ectopic homologous recombination, this may cause reciprocal translocations. Intrachromosomal homologous recombination may result in deletion events. When taking place between sister chromatids, DSB repair by homologous recombination bears a very low risk of introducing genetic alterations, and there are indications that sister chromatids are preferred to homologous chromosomes or ectopic regions of sequence homology, as donor of information [9, 10]. In contrast, end-joining mechanisms have a high potential for introducing genetic alterations during DSB repair. Whether or not end joining can restitute the original sequence, is to a large extent influenced by the conformation of the ends to be joined.

End-joining mechanisms and the function of the Ku proteins in end joining have been extensively reviewed (eg, [1, 11, 12]) and only an overview of the available information is given here. End joining was investigated on a molecular level in yeast, Xenopus, and a variety of mammalian cell lines. Mostly, plasmid rejoining assays were used where plasmids were linearized by restriction digest and then transformed/transfected into cells or incubated with cell extracts, followed by a determination of the relative frequency of joining and sequence analysis of the junction sites (eg, [13, 14, 15, 16, 17, 18]). In other investigations, chromosomal DSB were induced by site-specific endonucleases (eg, [14, 19, 20]). While there are organism-specific differences with regard to the joining efficiency of various end conformations (such as complementary or noncomplementary single strand protrusions and blunt ends) and the degree of dependence of these joining reactions on Ku, the following general picture emerges: provided that the conformation of ends allows for restitution of the original sequence (eg, in the case of complementary overhangs), Ku-dependent end joining is a rather accurate process in mammalian cells and *S cerevisiae* [13, 16, 17] (but not in *S pombe* [18]). In the absence of functional Ku proteins, alternative end-joining mechanisms are used that appear to largely depend on base pairing between regions of microhomology (ie, one to several bases of homology) exposed by single strand resection or unwinding of the double strand. Microhomology-dependent joining is

associated with the generation of deletions at the break sites and it has been proposed that Ku is required for protection of ends against nucleolytic attack. The deletion sizes may, however, also reflect positions of microhomology that allow stable strand-annealing.

When DSB are induced by radical attack (eg, after ionizing irradiation), the ends do not necessarily terminate in complementary single-strand protrusions. To mimic this situation, in some studies, noncomplementary ends were produced by digestion with combinations of restriction enzymes. Joining of noncomplementary ends is intrinsically an inaccurate process that will lead to sequence alterations, but again Ku-independent joining of these ends relies more on microhomology-mediated base pairing associated with deletion formation than does Ku-dependent joining [16, 17]. Current models propose that Ku serves as an alignment factor that facilitates joining reactions in the absence of sufficiently stable base-pair interactions between the ends to be joined, thus minimizing the need for end processing and, therefore, the nucleotide loss at the junction site [17]. Recently, it was also shown that the Ku-dependent process in vitro mediates accurate joining of complex DSB with partially cohesive overhangs terminating in 3′-phosphoglycolate, a substrate expected to occur upon radical-induced DNA breakage [21].

While Ku-independent end joining was observed in all organisms studied so far, its efficiency seems to vary between organisms. In yeast, inactivation of Ku drastically reduces the end-joining frequency, suggesting that Ku-independent mechanisms are rather inefficient. In contrast, in mammalian cells, the absence of functional Ku has little effect on plasmid joining frequencies, suggesting that Ku-independent mechanisms can take over efficiently for this kind of substrate [15, 16].

When judging the fidelity of end joining, we have also to consider the probability of mis-rejoining, that is, the joining of ends originating from different breaks. Mis-rejoining involves insertions of DNA fragments at break sites as well as interactions between chromosomal breaks that result in genomic rearrangements. In *S cerevisiae*, insertion of DNA fragments lacking homology to the genomic DNA is a very inefficient process, the efficiency of which can be increased by treating the cells with DSB-inducing agents such as ionizing radiation. This increase is not seen in Ku-deficient mutants, thus demonstrating a major role for the Ku-dependent end-joining mechanism in insertions occurring at break sites [22]. While nonhomologous integration in mammalian cells occurs readily, Ku-dependence of this process has also been shown by some authors (eg, [23]), but not by others (eg, [19]). Since the genetic requirements for Ku-independent end joining are not yet clear, its influence on repair-associated insertions remains to be elucidated. Extrachromosomal DNA fragments that may be inserted into chromosomal DNA in the course of DSB repair include pieces of organelle DNA and cDNAs [24], and possibly excised chromosomal fragments, or even foreign DNA. Hence, end-joining mechanisms may be involved in phenomena such as mitochondrion-to-nucleus transfer of genetic information, sequence duplications or horizontal gene transfer.

Treatment of cells with ionizing radiation or other strand-break inducing agents leads to chromosome rearrangements due to mis-repair of DSB. As mentioned before, they may result from cross-overs during homologous recombination between inappropriate sites or from mis-rejoining of chromosomal ends. Because of the redundancy of DSB repair mechanisms, the relative contribution of individual mechanisms to the generation of chromosomal rearrangements is difficult to analyze. In *S cerevisiae* mutant strains unable to perform DSB repair via homologous recombination, radiation-induced chromosomal aberrations (mainly exchange-type aberrations) occur with high frequency and their occurrence depends on functional Ku [25]. Similarly, radiation-induced exchange-type aberrations are considerably increased in the DT40 chicken B-lymphocyte cell line when homologous recombination is impaired by inactivation of RAD54, but not when KU70-dependent end joining is inactivated [4]. These data suggest a major role for the Ku-dependent repair process in the generation of repair-associated exchanges. In mammalian cells, however, the absence of functional Ku results in increased frequencies of spontaneous exchange-type aberrations, and inactivation of DNA ligase IV, which participates in Ku-dependent end joining, enhances the frequency of radiation-induced exchanges [26]. A possible explanation for these differences may lie in species-dependent variations in the efficiency of Ku-independent end-joining pathways that may also readily mis-rejoin chromosomal ends. Since mis-rejoining requires physical vicinity of ends resulting from different breaks, the number of breaks present at a given time in a cell nucleus may also affect the formation of exchanges. This number is affected not only by the dose of irradiation, but also by the kinetics of (correct) DSB elimination, thus complicating comparisons between different experimental systems. Nevertheless, a recent study, based on the restitution of full-length chromosomal restriction fragments after irradiation, demonstrated a dependence of mis-rejoining on the Ku-mediated process also in mammalian cells [27].

KU AND THE STABILITY OF CHROMOSOME ENDS

Telomeres are specific nucleo-protein structures at the ends of linear chromosomes (see [28] for a recent review). One of their functions is to enable template-independent elongation, thus allowing to overcome the end-replication problem. In most eukaryotes, elongation of the telomeric repeat tract is performed by telomerase. In addition, telomere-associated proteins form a so-called cap that protects the telomeres from degradation and recombination processes. This cap also enables the cell to differentiate between natural chromosome ends and unnatural ends caused by DSB, thereby inhibiting end-to-end fusions between chromosomes. This article concentrates on the influence of telomere-associated Ku on genome stability and the reader is referred to recent reviews covering additional aspects of Ku-dependent processes in telomere metabolism [28, 29].

When telomeric repeat DNA is lost in telomerase-deficient mammalian cells, fusions between chromosome ends are observed that lead to dicentric fusion chromosomes [30]. A concomitant loss of the proliferative capacity, the so-called cellular senescence, can at least partially be explained by anaphase bridges and breakage-fusion cycles. Some cells in senescent populations gain the capability of elongating their telomeres by telomerase-independent ALT (alternative lengthening of telomeres) mechanisms that probably depend on recombination between telomeric repeat tracts [31]. Recombination between subtelomeric repetitive sequence elements may also contribute to telomere stabilization in telomerase-deficient cells [32]. Telomerase-deficient yeast cell also undergo cellular senescence when the telomeric repeat tract is eroded; and survivors, which escape senescence, use mechanisms of alternative telomere maintenance that are similar to those observed in mammalian cells. Survivors in *S cerevisiae* exhibit strong amplification of the subtelomeric so-called Y′ elements or strongly elongated telomeric repeat tracts. Different recombinative subpathways appear to be responsible for Y′ amplification and telomere elongation in *S cerevisiae* survivor cells [28, 29]. In *S pombe* survivor cells, linear chromosomes, in which subtelomeric repetitive sequences are amplified, were observed. In addition, by end-to-end fusion, circularized chromosomes (rather than chromosome fusions) occur with high frequency [33].

In normal cells, recombination between telomeric or subtelomeric sequences and end-to-end fusions are suppressed. Recent research suggests that suppression depends not directly on the length of the telomeric repeat tract, but on the integrity of the proteinaceous cap. Ku bound to telomeres, either directly (as in *S cerevisiae*, [34]) or indirectly (as suggested for mammalian cells, [35]), appears to provide capping functions: in Ku-deficient mammalian cells, end-to-end fusions occur independently of the length of the telomeric repeat tract, demonstrating that Ku prevents such fusions and that these fusions are not produced by Ku-dependent end joining [36, 37, 38]. In Ku-deficient yeast cells, rearrangements and amplification of subtelomeric repetitive elements are observed, suggesting that Ku is involved in inhibiting recombinative processes in telomere-associated regions [39, 40]. Recombination is also thought to cause rabid shortening of artificially elongated telomeric tracks in Ku-deficient *S cerevisiae* [41].

KU AND THE MOBILITY OF RETROELEMENTS

Several recent studies show that the presence of functional Ku affects the outcome of retroviral infection in mammalian cells. Initial studies suggested a function for Ku (and other factors required in Ku-dependent end joining) in the repair of the gapped integration intermediate that results when retroviral integrase joins the 3′-terminal nucleotide of viral cDNA to staggered phosphates in the host DNA [42]. This view has subsequently been challenged, and it was proposed that Ku-dependent end joining may be required for circularization of unintegrated viral DNA (thus preventing apoptosis induced by the presence of viral DNA ends in host cells), or for repairing DSB induced in host DNA by unspecific endonucleolytic activity of free integrase molecules [43, 44, 45]. In support of the latter model, it has been observed that expression of HIV integrase is lethal in yeast mutants deficient for homologous recombination, the major DSB repair pathway in this organism [46].

The yeast *S cerevisiae* possesses retrotransposons (Ty elements) that resemble retroviruses and generate intracellular virus-like particles. Ku has been shown to physically associate with these particles [47] and with retroviral preintegration complexes in mammalian cells [45]. Interestingly, in *S cerevisiae*, the presence of Ku affects the substrate specificity of Ty integrase: when Ku-deficient yeast cells are transformed with linear plasmids lacking homology to the yeast genome, about 90% of the integration reactions depend on the expression of Ty elements, and the integrations show hallmarks of Ty integrase-mediated reactions. In contrast, in Ku-proficient cells, integration of plasmid DNA does not depend on Ty metabolism [22]. Ku, functioning as a cellular factor that determines integrase substrate specificity, may explain why integrase-mediated integration of Non-Ty-DNA was never observed in vivo, while it can readily be demonstrated in vitro [48].

CONCLUSIONS

The Ku proteins, as well as additional proteins involved in Ku-dependent end joining, are generally regarded as caretakers of genomic stability (eg, [49]) and indeed, increased chromosomal instability is seen when these proteins are inactive in mammalian cells. Ku-dependent end joining is a major DSB repair mechanism in mammalian cells, in the absence of which many breaks either remain unrepaired or are processed by other mechanisms that may cause chromosomal alterations. There are, however, indications that misrejoining of ends can also result from Ku-dependent DSB repair, leading to exchange-type aberrations or insertions of DNA fragments. The parameters that influence the probability of misrejoining are not yet known; while physical closeness is a prerequisite for interaction between DNA ends, it is unclear whether, in DSB repair, ends from different break sites meet incidentally or whether active processes can bring them together. The ability of Ku to join ends originating from different break sites is not surprising, given that Ku is involved in V(D)J recombination and class switch recombination of immunoglobulin genes. However, at least for V(D)J recombination, the situation differs from DSB repair in that the regions to be joined are juxtaposed before cleavage occurs within a protein complex involving the RAG proteins [50].

Even when the *correct* ends are joined in the course of DSB repair, the Ku-mediated mechanism clearly introduces genetic alterations more readily than homologous recombination between sister chromatids. These alterations are, however, in general smaller than those introduced by Ku-independent end joining. Thus, although Ku-dependent

DSB repair cannot be considered as an error-free mechanism, its general outcome appears to be less problematic than that of alternatives such as Ku-independent end joining or ectopic homologous recombination. A clear positive effect of Ku results from its function in the telomeric cap: Ku suppresses chromosomal end-to-end fusions and, at least in yeast, recombination events in telomeric and telomere-associated regions. It will be interesting to see whether Ku has also an influence on the occurrence of ALT mechanisms in mammalian cells. Additional positive effects of Ku on genomic stability may include suppression of integrase-mediated alterations. Thus, in spite of some negative effects, Ku can still be regarded as a caretaker of genomic stability.

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Journal of Biomedicine and Biotechnology • 2:2 (2002) 66–74 • PII. S1110724302202016 • http://jbb.hindawi.com

REVIEW ARTICLE

Answering the Call: Coping with DNA Damage at the Most Inopportune Time

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Received 25 January 2002; accepted 20 February 2002

DNA damage incurred during the process of chromosomal replication has a particularly high possibility of resulting in mutagenesis or lethality for the cell. The SOS response of *Escherichia coli* appears to be well adapted for this particular situation and involves the coordinated up-regulation of genes whose products center upon the tasks of maintaining the integrity of the replication fork when it encounters DNA damage, delaying the replication process (a DNA damage checkpoint), repairing the DNA lesions or allowing replication to occur over these DNA lesions, and then restoring processive replication before the SOS response itself is turned off. Recent advances in the fields of genomics and biochemistry has given a much more comprehensive picture of the timing and coordination of events which allow cells to deal with potentially lethal or mutagenic DNA lesions at the time of chromosomal replication.

MECHANISM OF INDUCTION

Irradiation of *Escherichia coli* with near ultraviolet light (254 nm UV) produces DNA lesions that block DNA replication and pose a dangerous threat to the integrity of the genetic information. DNA damage that blocks replication can result in genomic rearrangements when it resumes from the wrong place, mutagenesis when the incorrect base is incorporated opposite to the lesion, or even cell death when the block to replication cannot be overcome. A large body of work has demonstrated that *E coli* responds to this challenge by upregulating the expression of several genes which function to repair the DNA lesions, maintain the integrity of the DNA replication fork, and prevent premature cell division. This cellular response to DNA damage produced by UV and other agents has been collectively termed the SOS response, after the international distress signal (see [1]; reviewed in [2, 3]).

The sensor for SOS induction in *E coli* is a two component repressor/activator system of LexA and RecA that "senses" when the replication fork is impeded from progressing normally. The observation that nonreplicating *E coli* do not induce a strong SOS response following UV irradiation supports the notion that replication is necessary for signaling the response [4]. Many of the genes induced following DNA damage are regulated by the LexA repressor protein which binds to a 20 base pair consensus sequence in the operator region of the genes, suppressing their expression [5, 6]. Derepression of these genes occurs when RecA binds to single stranded regions of DNA created at replication forks disrupted by DNA damage. RecA bound to single strand DNA becomes conformationally active, serving as a coprotease that promotes the autocatalytic cleavage of the LexA repressor. As the cellular concentration of LexA diminishes, the genes normally suppressed by LexA are more frequently transcribed (see [2, 4] and the references therein).

Kenyon and Walker carried out the first systematic search to identify genes that are up-regulated in a *recA/lexA*-dependent fashion [7]. Through random insertion of a *lac* reporter gene into the *E coli* chromosome, they were able to identify promoters which were up-regulated following DNA damage. Subsequent analysis of the up-regulated genes revealed a 20 basepair consensus LexA binding motif, or "SOS box" shared by these genes in their promoter/operator regions [8]. Since these initial studies, several groups, using a variety of strategies, have identified many more LexA-dependent damage inducible genes, as well as some genes that are induced in a LexA-independent manner. [9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21]. In one recent approach, DNA microarrays containing amplified DNA fragments from 95.5% of all open reading frames identified in the *E coli* genome were used to examine the changes in gene expression during the first hour following UV exposure in both wild-type cells and *lexA1* mutants, which are unable to induce genes under LexA control [21]. These DNA microarrays contain PCR amplified DNA fragments of known and predicted genetic sequences printed on the surface of a glass slide. Through the comparative hybridization of two cellular RNA preparations, the relative difference between transcript levels of any gene in these preparations can be determined (Figure 1). These techniques, in total, identified 43 locations

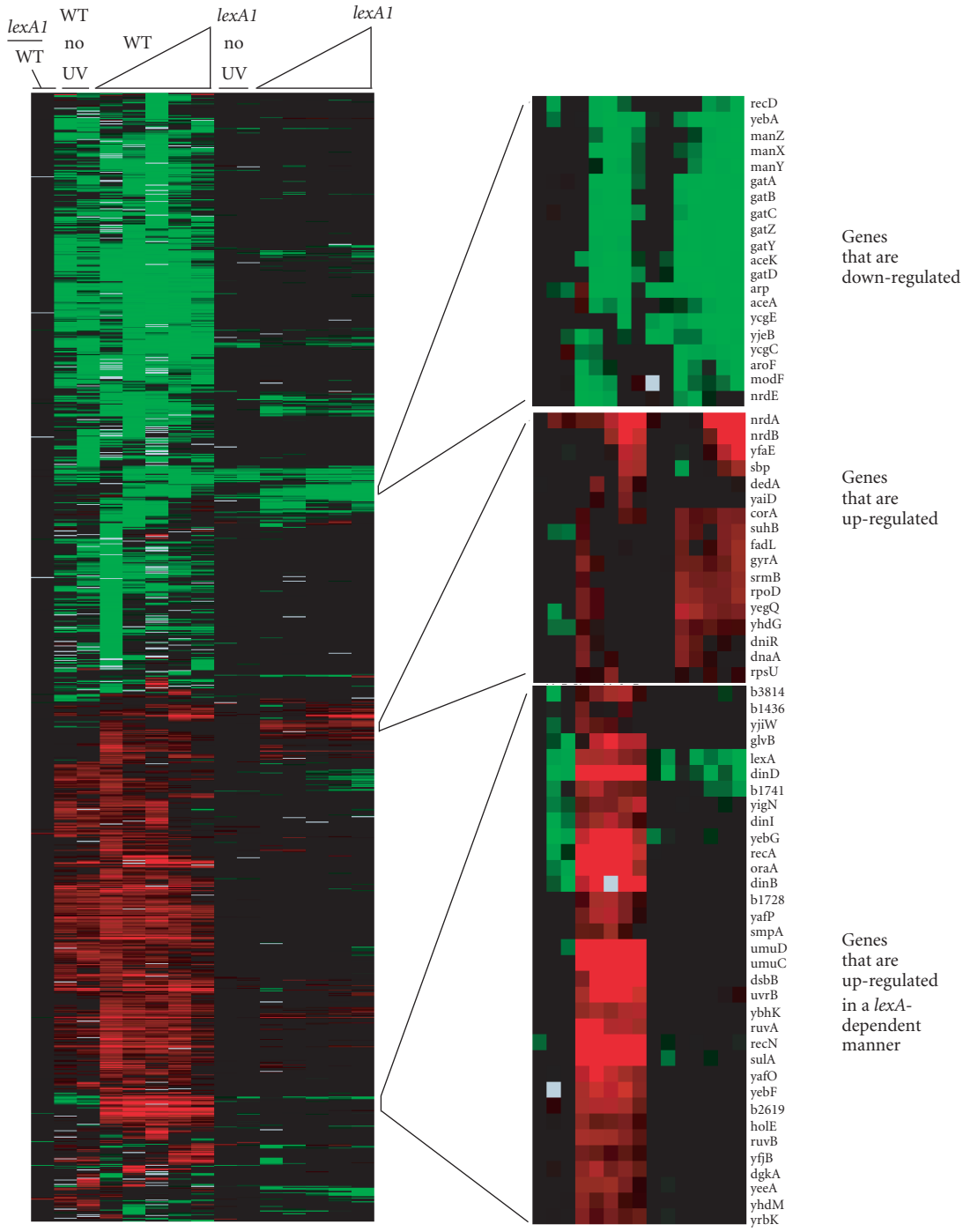

FIGURE 1. Clustered display of gene expression profiles following UV exposure in wild-type and lexA1 (SOS deficient) *Escherichia coli* [21]. Increased transcript levels are shown in red, decreased transcript levels are shown in green. The timepoints for irradiated samples (left to right) were 5, 10, 20, 40, and 60 minutes following UV irradiation. The timepoints for unirradiated samples (left to right) were 20 and 60 minutes following UV irradiation. All measurements are relative to time 0. Genes were selected for this analysis if their expression level deviated from time 0 by at least a factor of 2 in at least 3 time points. The colored image was produced using cluster analysis and is publicly available with this data at http://genome-www.stanford.edu/UVirradiation. The color scale ranges from saturated green for log ratios 2.0 and below to saturated red for log ratios 2.0 and above.

on the *E coli* chromosome that were up-regulated in a *lexA*-dependent manner after UV irradiation in actively replicating cells. In addition, several transcripts were either down-regulated or degraded following UV irradiation.

The biological function of many of these UV-responsive genes has been studied extensively and, in some cases, their role in the recovery process is well characterized. In this review, we outline some of the recent advances in our

understanding of the events that occur after the *E coli* genome is damaged, focusing particularly on the induction of cellular responses after UV exposure.

MAINTAINING THE REPLICATION FORK

One of the earliest recognized, and most heavily induced, DNA damage inducible gene products is RecA [22]. The act of binding to the single strand DNA regions at blocked replication forks serves not only to "sense" that replication is blocked, but also to maintain the structural integrity of the replication fork itself when progression is impeded. It is well established that *recA* mutants are extremely hypersensitive to DNA damage. This hypersensitivity correlates with a striking phenomenon called *rec*-less degradation in which the genomic DNA is rapidly degraded in the presence of DNA damage [22, 23]. The degradation is much more severe when replication is active in the cells at the time of damage and it has been shown to initiate at the replication forks and then degrade progressively back from these points. These observations led Horii and Susuki to propose that RecA plays a role in protecting DNA at replication forks when they are blocked by DNA damage [23].

recA was originally identified and characterized as a gene essential for recombination in *E coli* [24]. In vitro, purified RecA binds to single strand DNA progressively and then pairs that single stranded DNA with homologous duplex DNA. The product of this reaction creates a RecA protein filament bound to a three strand DNA structure (for reviews see [25, 26, 27]). During recombinational processes, this activity is thought to be critical for bringing together homologous strands from different DNA molecules. During DNA replication, this *same* biochemical activity of RecA may play a nonrecombinational role in maintaining the DNA replication fork [28, 29, 30]. Semiconservative replication copies both strands of the DNA template concurrently in a $5' \rightarrow 3'$ direction. While the leading strand can be synthesized continuously, synthesis on the lagging strand template occurs discontinuously, periodically reinitiating as the replication machinery moves processively along the template. The coordination of this process implies that at any given time, the region immediately behind the replication machinery will contain a single stranded region. In the event that replication becomes blocked or contains single strand gaps, the region left behind the replication fork should be an ideal substrate for RecA to bind and protect.

Several aspects of RecA function at replication forks arrested by DNA damage remain to be characterized. For instance, the structure of the blocked replication fork is not well understood. Does it end with the leading strand facing a lesion? Can the lagging strand continue on without the leading strand? It is also not known whether RecA promotes recombination at blocked replication forks, whether it is primarily nonrecombinational at these sites, or whether it is some combination of these two scenarios that depends upon the lesion presented. Finally, during recombinational processes, the act of RecA pairing single stranded DNA with homologous duplex DNA can also be associated with strand exchange and branch migration. These processes have also been proposed to function at damage blocked replication forks, perhaps to allow nucleotide excision repair or translesion DNA polymerases to gain access to the blocking DNA lesion [28, 29, 30, 31]. In vitro experiments suggest that several branch migration proteins are capable of acting on these substrates [32, 33, 34, 35]. However, whether branch migration occurs and which proteins, if any, act on replication structures in vivo has yet to be examined.

INDUCING REPAIR TO CLEAR THE DAMAGE

The production of DNA damage in the genome must be countered with a repair response that clears the DNA prior to the resumption of DNA replication and cell division. In *E coli*, UV-induced lesions are subject to nucleotide excision repair (NER), catalyzed by the UvrABCD proteins (reviewed in [2]). Three of these genes, *uvrA*, *uvrB*, and *uvrD*, are induced after DNA damage as part of the SOS response and have thus been implicated in promoting damage inducible excision repair. Recent studies have shed new light on the SOS-regulated NER response and have shown that inducible repair of UV lesions is dependent on the up-regulation of UvrA and UvrB but not UvrD.

Crowley and Hanawalt showed that cells treated with the transcription inhibitor rifampicin were deficient in their ability to remove the major UV-induced lesion, the cyclobutane pyrimidine dimer (CPD), from their genome following exposure to 40 J/m^2 UV [36]. A similar reduction in repair was observed in *lexA3*(Ind-) mutants that are unable to induce the SOS response after UV. However, *lexA51*(Def) mutants that constitutively express the SOS response were not deficient in NER even when treated with rifampicin after UV. Direct measurements of UvrA and UvrB levels in all three cell types correlated with the cell's ability to efficiently remove CPDs from the genome. In wild-type cells, 2-fold induction of both proteins occurred within the first 10 minutes after UV and maximal induction (as compared with the *lexA*(Def) strain) was reached within the first 40 minutes after UV, a time at which more than 80% of all CPDs are removed. Wild-type cells treated with rifampicin and the *lexA3*(Ind-) cells maintained low constitutive levels of UvrA and UvrB an hour after UV exposure, consistent with their slower rate of removal of CPDs. The pyrimidine(6-4)pyrimidone photoproduct (6-4 photoproduct), a less prevalent but more distorting UV-induced lesion, was repaired equally well in the presence or absence of SOS induction, indicating that inducible NER is necessary for efficient genomic repair of CPDs, but not 6-4 photoproducts in wild-type cells [36].

To determine the role of UvrD, also known as Helicase II, in promoting SOS-dependent inducible NER, Crowley and Hanawalt studied repair and survival in a *uvrD* deletion strain that carried a plasmid encoding a wild-type copy of *uvrD* under control of the T7 promoter. This plasmid construct yielded approximately wild-type levels of UvrD in an undamaged transformant and promoted almost complete complementation of the UV sensitivity and repair deficiencies observed in the nontransformed deletion mutant.

Since the levels of *uvrD* transcript could not be increased from the plasmid promoter after UV, the authors concluded that constitutive levels of UvrD are sufficient for promoting SOS-dependent NER in vivo [37]. It is interesting, therefore, to speculate on a role for UvrD in promoting cellular recovery from UV that is distinct from its role in NER. The observation that *uvrD* mutations are incompatible with polA (DNA pol I) or *rep* (Rep helicase) mutations suggests that Helicase II may function in replication in vivo [38, 39]. Recent genetic and biochemical investigations have provided further support for such a role [40, 41, 42, 43]. Its slow rate of up-regulation and modest 2.5-fold induction after UV [21] supports a role for UvrD that occurs later in the damage recovery process, perhaps after the majority of repair has been completed but before replication resumes.

An exciting new finding regarding the inducibility of *E coli* NER has come from the work of Moolenaar et al who have discovered a second endonuclease, dubbed Cho (UvrC homolog), that catalyzes a UvrB-dependent 3'-incision at a variety of lesions in the presence or absence of UvrC (43a). Cho, the gene product of the *ydjQ* gene, was discovered on the basis of its homology with the N-terminal region of UvrC, which is required for its 3'-incision activity. The *ydjQ* gene is a member of the SOS regulon and its gene product is up-regulated with kinetics similar to that of UvrA and UvrB [21]. Given that UvrC is not induced by DNA damage, this discovery suggests that *E coli* may possess an SOS-dependent subpathway of NER involving the novel Cho endonuclease.

GETTING BY THE LESIONS

The SOS response also upregulates the expression of three DNA polymerases, Pol II (*polB*), Pol IV (*dinB*), and Pol V (*umuC*) in addition to the constitutively expressed Pol I and Pol III. While Pol I, involved in repair and Okazaki fragment removal, and Pol III, the replicative DNA polymerase, can polymerize and incorporate the standard nucleotides opposite to their complementary bases, these inducible polymerases have the ability to incorporate and pair nucleotides opposite to specific forms of damaged DNA bases (ie, they promote translesion DNA synthesis). In vitro studies on single strand templates have shown that Pol III, the replicational polymerase of *E coli*, is arrested at DNA lesions such as those produced by UV irradiation [44, 45]. Several studies identified three DNA damage-inducible polymerases in *E coli* (as well as multiple homologs in eukaryotic cells, including XP-V) capable of incorporating nucleotides opposite DNA lesions with relatively high efficiency when Pol III had extended the template up to the blocking DNA lesion [9, 16, 44, 46, 47, 48, 49, 50]. Presumably, this allows lesions that would otherwise be blocks to DNA replication, a lethal event, to be bypassed and for replication to recover, a mutagenic event at worst. In fact, the first *E coli* translesion DNA polymerase, Pol V, encoded by *umuC*, was originally identified by its ability to produce mutations following UV irradiation, long before it was known to be a DNA polymerase [9]. In the absence of *umuC* (or *umuD*, see section: DNA damage replication checkpoint), the mutation rate drops dramatically following UV irradiation. Perhaps as a trade off for not mutating, these mutants are slightly more sensitive to UV irradiation than wild-type cells [9].

Following up on sequence similarities between the bypass DNA polymerase, REV1, identified by Nelson and colleagues [51] in yeast, both *umuC* and *dinB* were shown to be polymerases as well [47, 48]. Pol II, the product of the *polB* gene, was first identified as a polymerase and later shown to be inducible following damage, but its function has remained elusive since *polB* mutants are not sensitive to UV, which is sometimes interpreted to indicate a role in recovery of replication from DNA damage [13, 52, 53, 54]. Furthermore, no obvious replication defects are observed in *polB* mutants [55]. More recently, work from Rangarajan and colleagues [56] has shown that both the recovery of replication and the mutation spectra following UV irradiation is altered in *dinB* mutants, strongly suggesting that Pol II functions during the recovery process in vivo.

When and how these polymerases act at a DNA lesion in vivo remain unclear. Recent work from Napolitano and colleagues suggests that sometimes a polymerase might be specific for a specific DNA lesion but other times multiple polymerases may compete for the same DNA lesion, depending upon the nature of the DNA lesion and its sequence context [57]. Using a mutation assay based upon a plasmid construct that contain either an N-2-acetylaminofluorene (AAF) adduct, a benzo-a-pyrene adduct (BaP), or a 6-4 photoproduct, they demonstrated that the mutation frequency and spectra were altered in the absence of a specific bypass polymerase for each lesion. For instance, by measuring AAF induced −2 frameshifts, they found that mutagenesis was significantly reduced in *polB* mutants, but not in *dinB* nor *umuC* mutants. Conversely, when they measured BaP induced −1 frameshifts, they found that mutagenesis was significantly reduced in *dinB* and *umuC* mutants but not in *polB* mutants. This implies that, in vivo, different polymerases must be acting on different DNA lesions [57]. If and how the cell knows which polymerase to use at different DNA lesions remains an interesting but unknown question.

DNA DAMAGE REPLICATION CHECKPOINT

One of the bypass polymerases, Pol V (*umuC*) has an accomplice, *umuD*, which comprise an operon that is up-regulated rapidly following SOS induction. Since the initial discovery of these genes in screens for mutants that were not mutable by UV [9], extensive genetic and biochemical work has shown that they are required for error-prone translesion synthesis in *E coli* [48]. Following SOS induction, UmuD is proteolytically cleaved to form UmuD' by a mechanism similar to the LexA cleavage reaction [58, 59, 60, 61]. The proteins form a UmuD'$_2$C complex that is believed to be the active form promoting lesion bypass during replication recovery after UV damage. The uncleaved form of UmuD can also form an UmuD$_2$C complex that has been postulated to aid in the cessation of DNA

replication following the detection of UV-induced lesions in the genome [61, 62]. The UV dose-dependent cleavage of UmuD, the checkpoint protein, to UmuD', the bypass protein, occurs with kinetics that parallel the rate at which nucleotide excision repair proteins remove the bulk of UV-induced lesions following irradiation [36, 63]. This processing time is proposed to act as a checkpoint that contributes to cell survival by allowing ample time to repair the genome prior to recovery of replication, avoiding the production of more deleterious forms of DNA damage which would result from further attempts at replicating a damaged DNA template (reviewed in Smith, 1998 and Sutton, 2001).

The checkpoint role of UmuD comes from the initial observation that constitutive over-expression of the uncleaved form of UmuD, but not the cleaved form UmuD', results in a cold sensitive phenotype and a modest inhibition of DNA synthesis [64]. Opperman and colleagues have since shown that expression of noncleavable Umu(S60A)D and UmuC from a plasmid partially complemented the UV sensitivity of a Δ*umuDC* strain and delayed DNA synthesis and cell growth following UV. As predicted, a Δ*umuDC* strain resumed DNA synthesis and cell growth within the first 10 minutes after UV, whereas deletion mutants complemented with *umuC+umuD+* on a plasmid delayed resumption of replication for more than 45 minutes following 25 J/m^2 UV. Complementation of a Δ*umuDC* strain with a *umuD(S60A)C+* construct delayed DNA synthesis and cell growth over 60 minutes. Neither *umuD'C+* nor *umuD+C125* complemented the rapid resumption of DNA synthesis, despite the ability of both strains to perform translesion synthesis. It is important to note that resumption of replication was not observed in the *umuD(S60A)C+* construct, suggesting that UmuD'-dependent translesion synthesis is required for proper resumption of replication after UV [63, 65, 66].

To perform their roles in replication inhibition and translesion synthesis in vivo, the $UmuD_2C$ and $UmuD'_2C$ complexes have been shown to interact with DNA polymerase III directly, but each in distinctive ways. Using affinity chromatography, Sutton et al demonstrated that $UmuD_2$ has a greater affinity for the beta (processivity) subunit whereas $UmuD'_2$ interacts most strongly with the alpha (catalytic) subunit of Pol III. Both the UmuD and UmuD' homodimers bound to the epsilon (proofreading) subunit of Pol III [67]. Consistent with these interactions are data showing that only over-expression of the epsilon and beta subunits, and not any of the other eight Pol III subunits, had an effect on the cold sensitive phenotype of a *umuD+C+* over-expressing strain at the restrictive temperature [68]. Together, these data suggest that $UmuD_2C$ associates with the DNA replication complex directly and that these interactions serve to slow or block DNA synthesis, perhaps through sequestration of these subunits from the Pol III holoenzyme. It is interesting to note the similarity between this potential prokaryotic DNA damage checkpoint and the p21-dependent DNA damage inducible cell cycle checkpoint in eukaryotes. In eukaryotes, p21 targets PCNA, the eukaryotic analog of the beta subunit, to promote replication arrest after DNA damage [69].

DNA DAMAGE CELL DIVISION CHECKPOINT

E coli cells exposed to UV or other SOS-inducing treatments continue to elongate but fail to septate and thus grow as filaments. This inhibition of septation is an SOS-dependent process that involves the *lexA+*-regulated gene, *sulA* (*sfiA*). This gene was originally isolated as a suppressor of *lon* protease mutants, which are particularly prone to damage-induced filamentation [70]. The *lon* gene product is an ATP-dependent protease that degrades SulA in vivo. This degradation is rapid and, combined with LexA repression, serves to maintain SulA at low concentrations in the cell. Upon damage to the genome, *sulA* transcription is up-regulated dramatically, achieving 10-fold induction in the first five minutes after 25 J/m^2 UV [21]. Therefore, SulA accumulates to inhibitory levels only when the SOS response is induced. The *sulA* gene product inhibits septation by interacting with FtsZ, a key cell division protein that forms the septation ring early in cell division [71]. Evidence exists that a second gene, *sfiC*, also works to inhibit cell septation after DNA damage through interaction with FtsZ. This protein is DNA damage inducible but its regulation is not dependent on *lexA+recA+* [72, 73, 74].

Recently, two papers have suggested that a third mode of SOS-dependent septation inhibition exists in *E coli*. This mode of inhibition, is independent of *sulA* and *sfiC*, yet requires *umuDC* [65, 75]. In their studies of cold sensitivity induced by UmuDC overproduction, Opperman and colleagues noted the production of cell filaments at 30°C (the restrictive temperature) when *umuDC* were expressed constitutively from a single chromosomal copy of the operon. Over-expression of *umuDC* led to longer filaments and the production of filaments at 40°C. This role of UmuDC is apparently distinct from its role in cold sensitivity as physiological levels of the proteins induced cell division inhibition but not cold sensitivity. Unlike the cold sensitive phenotype, *umuDC*-dependent septation inhibition requires SOS induction, demonstrating the need for at least one other LexA-regulated gene in this process [65]. It is tempting to speculate that this gene might be *ftsK*, an essential cell division protein transcribed from *dinH*, an SOS-regulated promoter [76, 77]. It is unclear, however, how up-regulation of a gene required for septation would operate to inhibit this process after DNA damage. Perhaps high levels of FtsK prevent proper formation of the septation ring, perhaps by disrupting the stoichiometry of septation factors. Interestingly, over-expression of *ftsQAZ* from a plasmid suppressed *umuDC*-dependent filamentation [65]. This finding, combined with the recent demonstration that FtsK is required for recruitment of FtsQ and other factors to the septation ring [78], suggests that proper stoichiometry of septation proteins may indeed play a role in promoting the regulation of cell division in vivo. It is also possible that the UmuDC complex plays a direct inhibitory role in this process. Since it is the target of SulA and SfiC, FtsZ would be a logical target for inhibition by the UmuDC complex, but this interaction has yet to be demonstrated. There may also be a role for *minCDE* in regulating cell division and septation after DNA damage. These genes,

which induce and regulate septum formation, are repressed following UV in a *lexA*-independent manner [21]. Clearly, further investigation of this division inhibition is warranted, especially in conjunction with the role of UmuDC in promoting the arrest of DNA replication after DNA damage.

RELIEVING STRESS: TURNING THE SOS RESPONSE OFF

Thus, the SOS response appears to upregulate genes that hold replication and cell division in place until the offending lesions are repaired (or polymerases that allow replication to occur over the lesions are expressed). Once this has occurred, the stress response must be turned off. Two mechanisms that appear to play a role in returning the cell to normalcy are the LexA repressor and the product of the *dinI* gene.

LexA represses its own gene, and following SOS induction, LexA repressor is heavily induced [79]. In the current model, as long as replication remains blocked and single stranded DNA is present at the replication fork to activate and bind RecA, the LexA produced will be cleaved and SOS genes will continue to be expressed (reviewed in [2]). Once replication has resumed and the single stranded regions have been filled in, RecA no longer remains in the activated state and the LexA protein accumulates, binds, and represses genes that contain LexA binding sites at their promoters. Eventually, enough LexA is produced to repress all genes under its control and the cell resumes replicating and dividing normally.

Once replication has resumed and repair has been completed, there still remains an excessive amount of RecA in the cell due to its large up-regulation following SOS induction. One additional gene induced during the SOS response, *dinI*, has recently been shown to help eliminate excess RecA and help terminate the SOS response through an unusual mechanism. DinI was discovered as an SOS inducible gene through the original screen by Kenyon and Walker [7]. The first hint that DinI may play a role in suppressing SOS response came from work by Ohmori and coworkers who demonstrated that it could suppress the cold-sensitive phenotype of an unusual *dinD68* mutation. The *dinD68* allele causes SOS induction without any DNA damaging agent at temperatures less than 20°C through a mechanism that is not yet understood [80]. However, subsequent work from Yasuda's group [81, 82] additionally showed that DinI, when over-expressed, conferred a UV sensitive phenotype to cells, inhibited induction of SulA, and impaired LexA and UmuD cleavage following DNA damage. These are all activities that are consistent with repression, rather than activation of SOS functions. Recently, Camerini-Otero and coworkers have shown that the down-regulation occurs directly through DinI binding to the RecA protein itself [83, 84]. This group demonstrated that the C-terminal alpha helix region of DinI has an unusually strong negatively charged surface that appears to mimic the structure and charge of single-stranded DNA, the substrate for RecA activation. However, unlike RecA bound to single stranded DNA, RecA bound to DinI is not an activated form and cannot induce the SOS response. Thus, these data strongly suggest that DinI functions to down-regulate the SOS response by titrating away the inducing "sensor" for SOS induction itself, the RecA protein.

SUMMARY

The picture emerging from these studies of *E coli* is one in which multiple events are choreographed spatially and temporally such that the entire process can restore and complete the duplication of the genome following a moderate dose of DNA damage without sacrificing viability or the integrity of the genetic information.

There are, however, some critical pieces to this picture which remain to be filled in. It will be interesting and important to now identify the specific structure of the replication fork when it arrests. We believe that single stranded DNA is produced (and activates RecA) to initiate the cascade, however, where it is formed and how the arrested replication fork is structured remain to be characterized. Furthermore, the timing and contribution that lesion repair, lesion bypass, or even recombination has on the recovery of replication is not at all clear and remains a critical question since each strategy has very different consequences for the integrity and fidelity of the genetic information as it is duplicated. Lastly and most importantly, when we look at the genes that are induced following DNA damage, fully half of these genes have not been well characterized as to their functions. While new information is constantly coming, it is apparent from recent studies reviewed here that our view to date is extremely DNA-centric and is limited by a small number of assays. It will be interesting to see which discoveries remain to be elucidated from the characterization of these unknown genes.

ACKNOWLEDGMENT

This work was supported in part by an award from the National Science Foundation MCB0130486 to J. Crowley.

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Journal of Biomedicine and Biotechnology • 2:2 (2002) 75–85 • PII. S1110724302204052 • http://jbb.hindawi.com

REVIEW ARTICLE

Homologous Recombination and Its Role in Carcinogenesis

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Received 9 January 2002; revised 29 April 2002; accepted 29 April 2002

Cancer develops when cells no longer follow their normal pattern of controlled growth. In the absence or disregard of such regulation, resulting from changes in their genetic makeup, these errant cells acquire a growth advantage, expanding into precancerous clones. Over the last decade, many studies have revealed the relevance of genomic mutation in this process, be it by misreplication, environmental damage, or a deficiency in repairing endogenous and exogenous damage. Here, we discuss homologous recombination as another mechanism that can result in a loss of heterozygosity or genetic rearrangements. Some of these genetic alterations may play a primary role in carcinogenesis, but they are more likely to be involved in secondary and subsequent steps of carcinogenesis by which recessive oncogenic mutations are revealed. Patients, whose cells display an increased frequency of recombination, also have an elevated frequency of cancer, further supporting the link between recombination and carcinogenesis.

INTRODUCTION

Genetic alteration is the fundamental underlying process that allows a normal cell to evolve into a cancerous one. Genetic alterations can take a variety of forms with the essential result being that a gene, or a combination of genes, is altered to produce a cell that can bypass normal growth restrictions. Here, we present a body of evidence indicating that one of the important processes of genetic alteration in the generation of cancers is homologous recombination (HR). Evidence from our laboratory, and many others, have demonstrated that certain genetic deficiencies result in higher than normal levels of genomic instability including a higher frequency of HR. Patients with such genomic instability have a higher probability of developing cancers as the instability allows a higher rate of genetic alteration. These alterations may result in either the direct mutation of an oncogenic gene or, more likely, it reveals an already mutated copy. In addition, we present evidence that proliferating cells demonstrate the highest propensity for HR, in effect this predisposes proliferating cancer cells to an increased frequency of this form of genomic instability.

MODELS OF CARCINOGENESIS

Here, we mention three commonly accepted models of carcinogenesis to highlight some of the processes that may involve an HR event. The simplest model for carcinogenesis is a one-step event. Most often, a mutation occurs in an oncogene that acts dominantly allowing oncogenesis. Examples of oncogenes include c-ABL1, H-RAS, c-MYC, c-ERBB, v-FOS, and *c-JUN* [1]. Alternatively, the one-step model involves an inherited recessive defect that is exposed by the mutation of its functional counterpart, though actually, this "mutation" is most often a loss of heterozygosity (LOH) event. These recessive mutations are usually in genes classically called tumour suppressers (for a review see [2]).

A simple two-step model allows for the majority of tumour suppresser genes being present as two functional copies, where both copies have to be mutated to incapacitate functionality [3]. In the published literature, LOH is the most commonly reported event, as opposed to mutational heterozygosity. Recombination, be it by deletion of the functional allele or gene conversion of the functional allele into the mutated one, is the most likely mechanism for LOH, this is discussed further later in this review.

A multistep scenario has intriguing implications. Here, the initial mutation is the result of a DNA repair or metabolism defect. Such cells may accumulate somatic mutations at a higher frequency or may have a higher level of gross genomic instability. Those patients with a predisposition to genomic instability have a much higher incidence of cancer than the general population, and they have a much earlier onset of certain tumor types. Some of these diseases are outlined later in the review.

As yet it has been difficult to determine which gene is initially mutated in most cancers. The reason is two-fold, firstly, the majority of tumours display heterogeneity [4, 5, 6, 7], often with an associated genetic instability [8, 9, 10, 11]. This phenotype may be facilitated by the initial mutation being of a DNA repair gene (see section *Genetic instability syndromes* below, for reviews see [6, 7]). Secondly, not all the genes

that are involved in carcinogenesis have been identified. However, it does appear that several cellular pathways are often altered to produce the necessary changes that produce a cancerous cell.

HOMOLOGOUS RECOMBINATION IN MAMMALIAN CELLS

Homologous recombination in mammalian cells is often considered to be less prevalent than an alternative recombination pathway, namely, nonhomologous end-joining (NHEJ) [12]. Thus, as a process of DNA repair and carcinogenesis, HR has often been overlooked [13]. This idea is widely accepted as it is well known that a large proportion of the mammalian genome contains repetitive DNA sequences [14]. *Contrarily*, recent studies have shown that mammalian cells are in fact quite proficient in HR; Liang et al [15] demonstrated that a site specific break between two copies of a gene will result in homologous deletion at a relatively high frequency (30% to 50%). Further, the author of [16] determined that sister chromatid exchange is highly prevalent [16], followed by homologous interchromosomal recombination and then by ectopic recombination [17, 18]. In the last decade we, amongst several other researchers, demonstrated that deletions can be mediated by HR between repeated DNA fragments [19] and that the frequency of these events are elevated following exposure to cancer-causing agents [20, 21, 22, 23].

HOMOLOGOUS RECOMBINATION IN CARCINOGENESIS

Homologous recombination may be playing a fundamental role in carcinogenesis. In the following sections we outline six situations where HR may have a fundamental part to play in the progression to cancer. Firstly, we believe that the HR can be a major mechanism in the LOH, fulfilling the second step of the two-step model or a later event in the multistep model. Secondly, there are some cancer prone diseases that have genetic instability as a phenotype, some of these diseases also display an elevated level of HR. An increased frequency of HR makes it more likely that the LOH will occur at an accelerated rate, but also raises the possibility that HR will cause aberrant genomic rearrangements that may act as the primary step towards carcinogenesis. We also present some recent evidence that HR is more prevalent in proliferating cells. Together, these arguments provide compelling evidence that HR may be an important factor in the multiple steps required for carcinogenesis.

Mechanisms of loss of heterozygosity

There are various mechanisms that can result in LOH. Basically, the LOH results from one allele being lost from a cell that is then either homozygous or hemizygous for the remaining allele. Homozygosity can be attained when a gene conversion event occurs. Hemizygosity occurs when one allele is lost, as its DNA is no longer present in the cell. This latter event may occur by the deletion of the region containing the gene or during the division by chromosome loss.

Gene conversion [14, 24, 25] is a unidirectional transfer of information. In such an event, DNA is copied [26, 27] from one chromosome or chromatid to another without necessarily altering the arrangement of flanking markers. The frequency by which this HR mechanism occurs is difficult to determine as most gene conversions probably go undetected. Much of our understanding of this and other recombination mechanisms comes from analogous comparison to work performed in the model organism *Saccharomyces cerevisiae*.

Chromosome loss is a major mechanism of LOH. This type of event results in a deviation in the chromosome number to produce a cell that is aneuploid. It is interesting to note that almost every type of histological cancer carries cells with highly heterogeneous patterns of aneuploidy (for review see [28]). Once aneuploid, cells are often genetically unstable, as seen in cases of congenital aneuploidy. Patients with this congenital abnormality often display a high incidence of neoplasia (for reviews see [29, 30]).

A translocation is the transfer of a part of one chromosome to a nonhomologous chromosome. Translocations are often reciprocal, exchanging two different DNA segments. The break point of a translocation event may occur within a gene, thus destroying its function or altering its expression pattern, for example, the Burkitt lymphoma. One such translocation, the Philadelphia chromosome (chromosome 9/22 translocation), which produces a *BCR-ABL1* compound gene and results in chronic myelogenous leukemia. Two studies mapped the breakpoint of the Philadelphia chromosome and found that the translocation was mediated by a region of shared homology [31, 32] implicating HR as the mechanism.

There are three basic mechanisms that may produce a DNA deletion event (see Figure 1), the replication slippage, intrachromosomal and interchromosomal recombination. The replication slippage during DNA synthesis may produce a deletion, these deletions tend to be small [33, 34, 35, 36, 37] and most often occurin special regions where short tandemly reiterated sequences exist. The most common example of this is microsatellite instability, a phenomenon most prominent in hereditary nonpolyposis colon cancer. The causative mutations of this disease are in the mismatch repair genes resulting in a lack of replication proofreading [38, 39, 40, 41] and, therefore, an increased frequency of replication errors.

Intrachromosomal deletions are the result of aberrant recombination, many times mediated by regions of homology and can remove very large regions of DNA. Such deletions have been identified as the cause of several diseases, which include X-linked ichthyosis where 1.9 Mb, megabases, of DNA are deleted mediated by flanking homologous S232 elements [42, 43], hereditary neuropathy with liability to pressure palsies where 1.5 Mb are deleted mediated by CMT1A-REP [44, 45, 46] as well as Prader-Willi syndrome [47], DiGeorge syndrome [48], and hypercholesterolemia [49], all these examples are due to deletions mediated by HR between flanking regions of homology. There are several mechanisms that may produce an HR mediated intrachromosomal deletion, three of the most likely being an intrachromosomal crossover

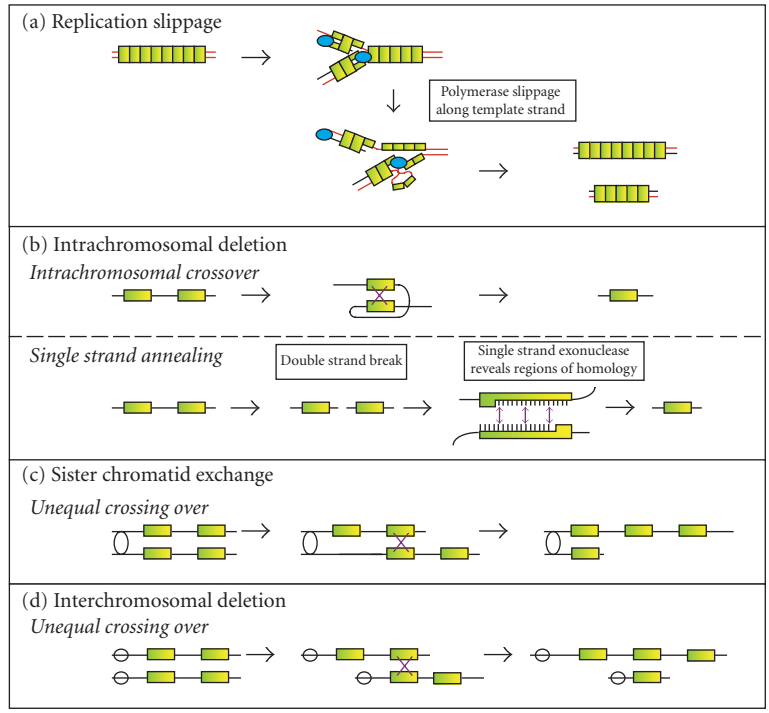

Figure 1. Mechanisms of deletion. (a) Replication slippage, where DNA polymerase dissociates from its template and reanneals to homologous sequences nearby resulting in either a deletion (shown) or insertion (not shown) of sequences. These tend to be relatively small deletions or insertions and are usually in regions of repetitive DNA. (b) Intrachromosomal or intrachromatid deletion may be mediated by a number of different mechanisms, two of the most likely being a crossover event and single strand annealing. A crossover event is mediated by aligning homologous sequences, strand invasion, possibly following a single-stranded break, allows strand exchange and recombination between the two homologous sequences. The result is a deletion of the intervening sequences. Single strand annealing is another likely mechanism that requires a double strand break between the homologous sequences. A single strand exonuclease can degrade one strand at the DNA ends until homology is revealed allowing the broken ends to anneal and the intervening sequences to be clipped off. (c) Interchormatid deletion is most likely to result from an unequal crossover event, only occuring in G2 after the chromatid has been replicated but before they are segregated. Again, the event is mediated by a repeated region of homology, but in these events two products are formed, a deletion and a triplication on the two resultant recombinant chromosomes. (d) Interchromosomal deletion is similar to interchromatid deletion except that the interaction is between homologous chromosomes.

event, single strand annealing (reviewed in [50]), or unequal sister chromatid exchange. Single strand annealing is initiated by a double strand break (DSB) in a nonhomologous region between repeats or within one repeat. DNA degradation of single strands from exposed 5′ ends of DSBs leads to single-stranded regions, which anneal with each other once the degradation has exposed the repeated sequences. The 3′ tails are processed and nicks are ligated, producing the deletion. Unequal sister chromatid exchange may occur during the DNA replication, probably initiated following the replication fork stalling [51]. A deletion results from an unequal crossing over between misaligned homologous regions on sister chromatids producing a deletion on one chromatid and a duplication of the same region on the other, these then segregate in the daughter cells that are produced. The final class of deletion is an interchromosomal event, this is very similar to an unequal sister chromatid exchange, except that the interaction is between homologous chromosomes or ectopic homologous regions and is not necessarily dependent upon replication. It should also be noted that the LOH by deletion can also be mediated by NHEJ. In these events, two ends of DNA are brought together by two or four bases of microhomology. Many of these types of event have been modelled in yeast [52] and human tissue culture cell systems [53].

Inter and intrachromatid recombination events are only distinguishable by the presence or absence of a reciprocal duplication product. In this respect, it is interesting to note that the Charcot-Marie-Tooth disease type 1A occurs from a duplication of the same region as is deleted in hereditary neuropathy with liability to pressure palsies [46, 54, 55]. Similarly, a tandem duplication within the *ALL-1* gene is mediated by *Alu* recombination and results in acute myeloid leukemia [56]. These duplications suggest that an interchromatid crossing over mechanism is responsible for these events.

Gene conversion, deletion, and perhaps translocation may be mediated by HR. In the past decade, we have used homologous deletion to detect genomic instability in a yeast model systems [57, 58], in human cells [22] as well as in vivo in mice [21, 23, 59]. Some of the most interesting results from these studies are presented later in this review.

Genetic instability syndromes

Assuming that genome rearrangements and deletion events cause a significant proportion of cancers, then there should be a correlation between those mutations that result in a higher recombination frequency and cancer predisposition. In fact, there are several genetic diseases that have a genetic instability phenotype and indeed have a high frequency of carcinogenesis. These include the Ataxia telangiectasia (AT) [60], Li-Fraumeni syndrome [61], Bloom syndrome [62], Werner syndrome [63], Cockayne syndrome, Fanconi anemia, Lynch syndromes I and II, Wiscott-Aldrich syndrome, and xeroderma pigmentosum [64]. Some of these diseases are presented in more detail below.

The Li-Fraumeni syndrome is a dominantly inherited disorder characterized by an early onset of cancer. The most prominent of these cancers are carcinoma of the breast followed by sarcomas, brain tumors, leukemia, lymphoma, lung carcinoma, and adrenocortical carcinoma, usually in children and young adults. The overall risk of cancer in these patients is nearly 100%, with over 50% of patients developing breast cancer by age 50 (reviewed in [65]). Li-Fraumeni syndrome patients, who carry a recessive mutation in *TP53*, have an exceptionally high risk of developing multiple primary cancers [66]. p53 has been proposed to be involved in maintaining the stability of the genome [61, 67, 68, 69, 70, 71, 72, 73, 74] by either its function in cell cycle arrest or apoptosis. At early passages, fibroblasts from *Trp53-/-* mice develop several chromosomal abnormalities [75]. Tumours from *Trp53-/-* mice are often aneuploid and there has been some evidence of chromosomal instability [76, 77]. In addition, p53 may inhibit HR via a putative interaction with the HR machinery protein RAD51 [71, 78]. How p53 is involved with HR is still not clearly understood, though many studies have undertaken to examine the relationship, most showing that cells lacking p53 have a higher than normal frequency of HR [71, 72, 73, 79, 80, 81].

Ataxia telangiectasia is an autosomal recessive syndrome. Among the phenotypes that patients display are chromosomal instability, radiosensitivity, and a predisposition to lymphoid cancer in childhood. Although AT is a relatively rare disorder, it has been estimated that about 1% of the general population is heterozygous for *ATM* mutations [82]. These heterozygous carriers may have a predisposition to sporadic breast cancer, though this correlation is still under discussion (for a review see [83]).

Cells from AT patients display chromosomal instability both spontaneously and following induction by ionizing radiation or radiomimetic agents (reviewed in [84, 85]). Cytogenetic analysis revealed a higher spontaneous incidence of chromosome breaks, chromosome gaps, acentric fragments, dicentric chromosomes, and aneuploidy. In addition, the T lymphocytes have an elevated frequency of translocations with break points mapping to the T-cell antigen receptor genes and the Ig heavy chain genes (reviewed in [85]). Following exposure to ionizing radiation or radiomimetic agents, cells from AT patients have an increased frequency of chromosomal aberrations compared to normal cells [60, 84, 85, 86]. In vivo, we have reported that Atm-deficient mice have an increased frequency of spontaneous HR [87]. In comparison, Turker et al demonstrated that, in the same mouse background, a deficiency in Atm did not result in an increased frequency of mutations [88], thus indicating that HR plays a more important role in the etiology of the AT.

ATM is generally thought to be important in activating p53 in response to the DNA damage [89, 90]. Recent reports have highlighted the multifunctional aspect of ATM, including that it phosphorylates BRCA1 [91, 92] and NBS [93, 94, 95, 96] following irradiation. In addition, there have been several reports linking ATM, through c-ABL1, to the HR protein RAD51 [97, 98, 99, 100]. Again, as with p53, how do these interactions relate to the HR is not fully understood, but it is an area of intense research.

A mutation in the *BRCA1* gene is estimated to confer a 70% risk of breast cancer by age 70 (reviewed in [101]). There have been numerous studies examining the frequency of breast cancers. From these studies, it is evident that mutations in the *BRCA1* and *BRCA2* genes result in an early onset of cancer and are responsible for a high percentage of premenopausal breast cancers (12% to 28%). The percentage depends on the prevalence of founder mutations within the population examined [102, 103, 104, 105, 106, 107, 108, 109, 110], the incidence of nonfamilial breast cancers tend to occur at a later age. Inactivation of *BRCA1* or *BRCA2* confers genetic instability such as aneuploidy and chromosomal rearrangements [111, 112, 113, 114, 115, 116]. In addition, both BRCA1 and BRCA2 play a role in HR, in the absence of either protein, HR repair of double-stranded breaks is defective [117]. It has been reported that BRCA1, BRCA2, and RAD51 form foci in the nucleus following the DNA damage [118, 119, 120] in an ATM-dependent manner [92].

RAD51, RAD52, and RAD54 are components of the RAD52 epistasis group [12, 121, 122], homologues of the genes defined in yeast to be necessary for an HR reaction. In vitro, it has been shown that RAD52 binds single-stranded tails at the sites of resected DSBs [123] as well as capping the exposed terminal nucleotide [124]. Both RAD51 and RAD54 form foci following the DNA damage [125]. In addition, the loss of RAD54 leads to recombinational deficiencies and DSB repair defects [126, 127]. The absence of RAD51 results in an accumulation of chromosomal abnormalities and cell death [128]. Both RAD51 and RAD54 have been shown to mediate sister chromatid exchange [129] and both form foci following exposure to ionising radiation, the kinetics of these foci are altered in ATM-deficient cells [130, 131]. How do the observed foci relate to the HR, is still unclear, but it does appear that BRCA1 is a component of several DNA damage response mechanisms [132] and may be responsible for activating HR in certain circumstances.

The genes mutated in Bloom's and Werner's syndromes, *BLM* and *WRN*, respectively, are highly homologous to RecQ helicase [133, 134], and were postulated to be involved in recombination. Cells from Bloom's syndrome patients show a high frequency of sister chromatid exchanges, hyper recombination, and chromosomal breakage. Patients with Bloom's

syndrome also show a greatly elevated predisposition to cancer of the sites and types that affect the general population [135]. Similarly, cells from Werner's syndrome patients show a 50-fold elevation in mutation rate, with the predominant form of mutations being gross DNA deletions [63]. The Werner syndrome patients age prematurely and show features like early onset of cataracts, generalized hair loss, loss of skin elasticity, osteoporosis, atherosclerosis, and short stature [136], they also often develop nonepithelial tumours and, to a lesser extent, leukemia, and carcinomata. These cancer prone diseases have in common a defect in genomic stability. Notably, both BLM and WRN have now been associated with processing the structures associated with stalled replication forks [137, 138], which may explain the observed phenotypes.

Fanconi's anemia (FA) is an autosomal recessive genetic disorder characterised clinically by progressive bone marrow failure, skeletal deformities, and a predisposition to neoplasia [139, 140]. Patient cells manifest an extreme chromosomal instability and hypersensitivity to polyfunctional alkylating agents. Most interstingly, cells from FA patients as well as cell extracts show a much elevated frequency of HR measured with plasmid constructs [141].

Although the AT has been identified to be the result of a mutation in the *ATM* gene, two other mutations result in syndromes that were originally mistaken to be AT. These variants of AT are caused by mutations in NBS (the syndrome is presently called Nijmegen breakage syndrome) [142] and in MRE11A [143], and present similar phenotypes, including genetic instability. NBS, MRE11, and RAD50 form a complex that NBS modulates once it is phosphorylated by the ATM in response to the DNA damage [93, 94, 95]. In yeast, it has been shown that RAD50 and MRE11 are involved in NHEJ [144, 145, 146], a mechanism that can repair double strand breaks and competes with HR. Assuming that the mammalian homologues of these genes are also involved in NHEJ, it seems plausible that a deficiency in ATM also results in a slight deficiency in NHEJ. Therefore, the damage would be channelled into HR as an alternative pathway, possibly explaining the hyper recombination phenotype that we found in Atm-deficient mice [87]. Most recently, it has been demonstrated that the WRN interacts with the Ku heterodimer [147], the complex thought to bind double strand break ends at the initiation of NHEJ [12, 148, 149, 150]. Thus, in a fashion similar to AT, a WRN deficiency may lead to an increased frequency of HR by default.

Susceptibility of proliferating cells to homologous recombination

Actively dividing cells are thought to be the most prone to developing cancer. Mitogenesis has been proposed to be an important contributor to carcinogenesis [151, 152] as evidenced by a higher risk for cancer after tissue regeneration. Furthermore, chemical carcinogenesis and transformation are most efficient if the target cells are treated just prior to or during the S phase [153, 154].

Using yeast to investigate the effect of the cell cycle arrest on the induction of deletions mediated by HR by different carcinogens, it was found that only DNA double strand breaks induce homologous deletion recombination in arrested cells, other forms of DNA damage such as DNA single strand breaks, UV lesions, as well as exposure to alkylating agents need DNA replication to induce homologous deletion recombination [155, 156].

As mentioned earlier, HR events are mediated by the RAD52 epistasis group. It is interesting to note that the protein and mRNA levels of this group tend to correlate with cell proliferation. For example, it has been reported that RAD51 expression is the highest in intestinal and uterine epithelia [157], which are highly proliferative. RAD51 has also proven to be essential in early mouse development [158, 159], a time of massive cellular proliferation. Consistent with the correlation with cell proliferation, both RAD51 and RAD54 are maximally transcribed in the S phase, during DNA synthesis [157, 160, 161, 162]. These observations are suggestive of a function for HR in proliferating cells, especially in combination with the damage inducibility of HR in proliferating cells. Takata et al [163], using chicken DT40 cells, demonstrated the involvement of NHEJ in G1 to early S phase, with HR functioning more in late S phase to G2. The role of replication was further demonstrated by Saintigny et al [164], who demonstrated that HR is increased in late S phase, only after the RAD51 foci formation. These studies strongly support the recent proposal that HR performs a special function during replication, namely, in resolving stalled replication forks [165, 166, 167, 168]. Altogether, it appears that HR is a common feature of the normal cell and may be especially harnessed by highly proliferative cancer cells.

CONCLUSIONS

In conclusion, we have presented a body of evidence that HR can play a role in different stages of carcinogenesis. While HR may contribute to the initial steps of carcinogenesis, we believe that HR functions mostly as a secondary or subsequent step in tumor progression. If genomic rearrangements and deletion events were the cause of a portion of the cancers, it might also be expected that certain carcinogens would increase the frequency of genome rearrangements. This has in fact been elegantly demonstrated in yeast [58, 169, 170], in human cells [22], as well as in vivo in mice [21, 23, 59]. With a wide variety of carcinogenic agents able to induce HR, it is easy to suggest that the normal day-to-day exposure to a variety of environmental and endogenous damages will also increase the frequency of HR. As can be observed in those patients who have an up regulated level of HR, an increased frequency of HR events can be highly deleterious. In addition, the sensitivity of proliferating cells to HR is highly correlative with proliferating cells being more prone to cancer and fits with current models of replication/recombination. Finally, the HR is likely to play a major role in producing the observed heterogeneity in many tumours. All in all, HR may be much more prevalent during carcinogenesis than previously considered.

ACKNOWLEDGMENTS

This work was supported by grants from the American Cancer Society No. RPG-95-076-04-MGO and the National Institute of Environmental Health Sciences, NIH, RO1 grant No ES09519 and KO2 award ES00299 (to R.H. Schiestl), and NIH RCDA Award No. F32GM19147 (to A.J.R. Bishop).

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Journal of Biomedicine and Biotechnology • 2:2 (2002) 86–93 • PII. S1110724302204027 • http://jbb.hindawi.com

REVIEW ARTICLE

Recombinational DNA Repair in Cancer and Normal Cells: The Challenge of Functional Analysis

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Received 29 November 2001; revised 28 February 2002; accepted 5 March 2002

A major goal of current cancer research is to understand the functional consequences of mutations in recombinational DNA repair genes. The introduction of artificial recombination substrates into living cells has evolved into a powerful tool to perform functional analysis of DNA double strand break (DSB) repair. Here, we review the principles and practice of current plasmid assays with regard to the two major DSB repair pathways, homologous recombination and nonhomologous end-joining. A spectrum of assay types is available to assess repair in a wide variety of cell lines. However, several technical challenges still need to be overcome. Understanding the alterations of DSB repair in cancers will ultimately provide a rational basis for drug design that may selectively sensitize tumor cells to ionizing radiation and chemotherapy, thereby achieving therapeutic gain.

INTRODUCTION

The past few years have seen an explosive increase in the understanding of both the molecular mechanisms and the genetic determinants of recombinational DNA repair. Mutations in genes controlling recombination lead not only to defective repair of DNA double strand breaks (DSBs) and hypersensitivity to ionizing radiation (IR), but also to genomic instability, developmental failure, and carcinogenesis [1, 2]. Particular excitement has been generated by the finding that several cancer susceptibility syndromes result from mutations in recombination-associated genes, including ataxia telangiectasia (*ATM*), Nijmegen breakage syndrome (*NBS1*), AT-Like disorder (*Mre11*), Fanconi Anemia (*FANC* genes), Werner Syndrome (*WRN*), and certain familial breast cancers (*BRCA1/2*) [1, 2]. Sporadic mutations and polymorphisms in a variety of genes involved in recombination have been found not only in normal tissues but also in malignant tumors, including *BRCA1*, *RAD54*, *RAD52*, and *XRCC3* [3, 4]. However, it is largely unknown what the functional significance of these alterations is. There is tremendous interest in a better understanding of the intricate protein networks in which these and other gene products cooperate. Undoubtedly, this knowledge will ultimately have significant implications for cancer prevention and treatment.

The task of characterizing the in vivo repair phenotype that results from any given spectrum of mutations in normal and malignant human cells is challenging. Several assays are available, including the determination of cellular sensitivity to genotoxic agents, cytological evaluation of repair-associated processes and molecular analysis of DNA repair products. Our laboratory has focused on the derivation and application of DNA plasmid assays, which have evolved as a powerful tool for the study of recombination in living cells. Here, we review the principles and practice of current plasmid assays with regard to the two major double strand break repair pathways, homologous recombination (HR), and nonhomologous end-joining (NHEJ). Due to space limitations, we are only able to consider a small number of studies and data sets. For details on mechanisms and genetic determinants of HR and NHEJ, the reader is referred to excellent recent review articles [1, 4, 5] (*including this issue of JBB*, *DNA Damage, Repair, and Diseases*).

HOMOLOGOUS RECOMBINATION

Chromosomal DSB repair assays utilizing the I-SceI endonuclease

The repair of a DSB by HR requires an undamaged template molecule that contains a homologous DNA sequence. Such a template can be provided by the sister chromatid, the homologous chromosome, or an adjacent repetitive sequence on the same chromosome. Specifically designed DNA plasmid substrates typically model homology-directed DSB repair by utilizing tandem repeats of a bacterial antibiotic resistance gene. Commonly, the 18 base pair recognition sequence for the rare-cutting I-SceI endonuclease is introduced into one gene copy, thereby inactivating gene function (for review [6]). The second copy is made inactive by other means. The repair substrate is stably integrated into the cell's genome (Figure 1a). After the introduction of a break at the I-SceI site, only a homology-mediated event can reconstitute the gene and confer cellular resistance to a selection antibiotic in cell tissue culture. The design of the tandem repeat and the I-SceI insertion site can be modified to allow for the

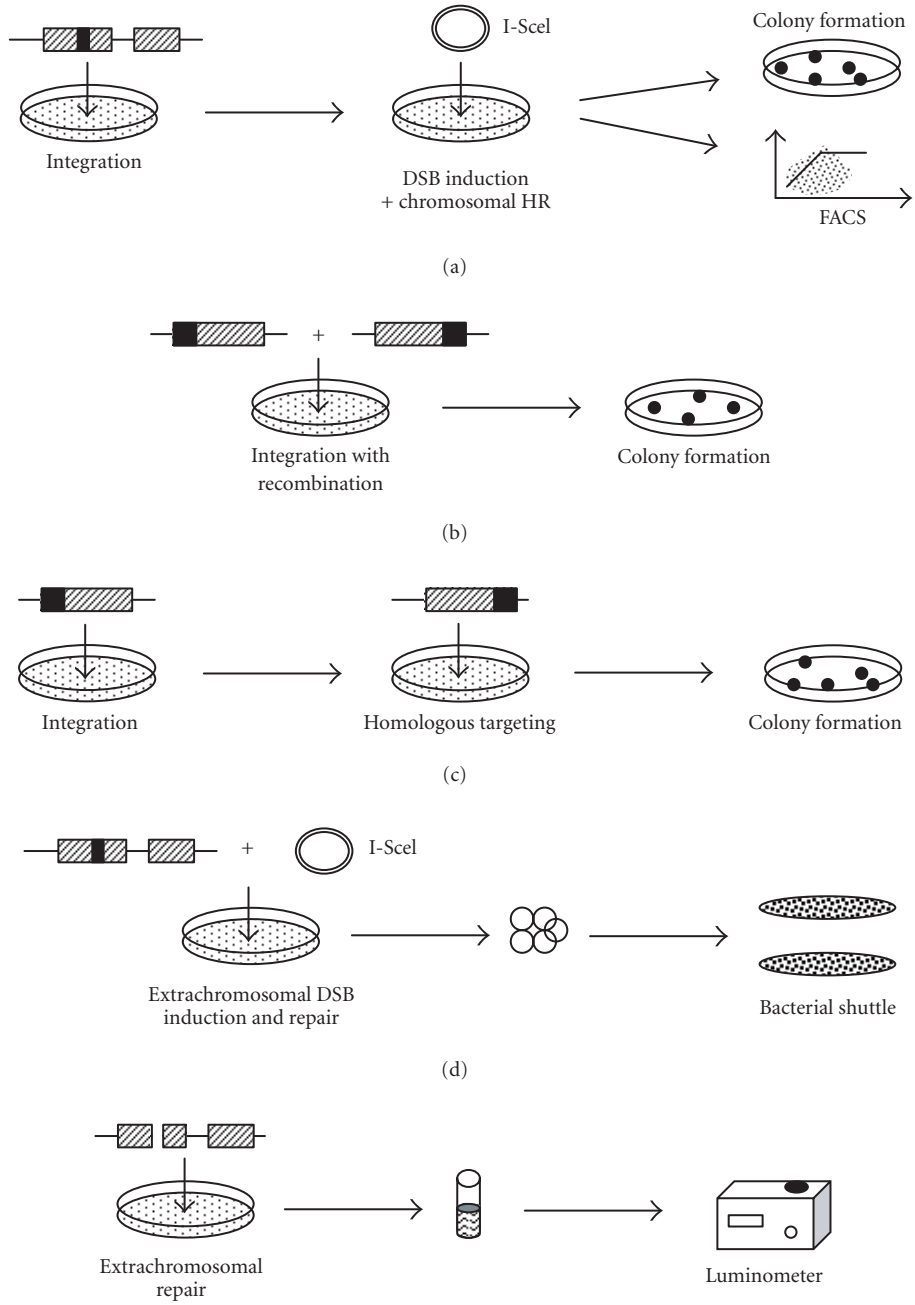

Figure 1. Illustration of the principles of measuring HR with plasmid assays. (a) Chromosomal DSB repair assay [14, 15]. A linearized plasmid substrate containing mutated resistance or marker genes is transfected and stable chromosomal integrants are selected, for example, using a puromycin resistance gene. Single-cell derived or pooled populations are amplified, and the integrated plasmid is characterized. Single or multiple copy integrants can be processed. The I-SceI expression vector is transiently transfected and usually 48 hours are allowed for the generation and homology-directed repair of I-SceI breaks within the integrated substrate (black insertion). The HR frequency can be assessed by selecting for the recombined antibiotic resistance gene in a colony formation assay (eg, *GPT*), or by assaying the marker gene product such as the green fluorescent protein (GFP) via flow cytometry (FACS). (b) Integration-associated HR [18]. The linearized substrates pΔ2 and pΔ3, each carrying a mutated *GPT* gene copy (black boxes, gene deletions), are cotransfected. HR before and during plasmid integration leads to the reconstitution of the resistance gene. Drug selection is used for isolation of stable recombinants in a colony formation assay. (c) Homologous gene targeting [29]. Linearized pΔ2puro is stably integrated using puromycin. Transient transfection of pΔ3 allows for HR between the chromosomal *GPT* gene copy and the extrachromosomal *GPT*. The reconstituted *GPT* gene is selected for using colony formation. (d) Episomal HR with replication [30]. An episomally replicating recombination substrate carrying mutated tandem repeats of a *CAT* reporter is transiently transfected together with an I-SceI expression vector. Breaks are generated within one *CAT* copy (black insertion) and repaired via HR using a downstream homologous template. After 48 hours, episomal plasmids are extracted and tested for the functional *CAT* gene in a bacterial shuttle vector assay. (e) Extrachromosomal HR. The mutated recombination substrate (*CAT* or *Luciferase* gene) is cut prior to transfection. HR reconstitutes the gene function, which can be measured by assaying cell extracts in a luminometer or scintillation counter.

assessment of genetically distinct subpathways of HR [7, 8]. Modifications of the plasmid substrate have permitted studying of interchromatid and even interchromosomal recombination [7, 9, 10]. Johnson and Jasin [11] recently reviewed the use of the I-SceI system in mammalian cells.

Many laboratories have employed the I-SceI assay, mostly in rodent cell lines [7, 8, 11, 12, 13, 14]. Several groups including our own have found up to 100-fold or more stimulation of spontaneous recombination frequencies upon the expression of the I-SceI enzyme. These studies have established that chromosomal HR can serve as a significant alternative to NHEJ in mammalian cells. The I-SceI assay has provided insight into the genetic determinants of HR. Reduced repair of I-SceI breaks has been observed in the absence of functional *RAD51*, *RAD54*, XRCC2, XRCC3, BRCA1, and BRCA2, and these findings have correlated with increased cellular sensitivity to IR [7, 8, 14, 15, 16, 17]. Thus, the repair of I-SceI breaks is at least partially carried out by the same protein network that is involved in the repair of radiation-induced DNA damage.

The ATM and p53 gene products, which are central proteins in the DNA damage response pathway, have been reported by us and others as being involved in the regulation of HR [18, 19, 20, 21, 22]. The *P53* gene has been observed to suppress spontaneous HR by at least one order of magnitude in a wide variety of rodent and human cell lines; and this could be a means by which p53 maintains genomic stability [23]. Mutation of *ATM*, which is upstream of *p53*, led to increased and error-prone HR (see below, section Plasmid assays containing extrachromosomal components) [18]. However, it has not yet been established whether and how *ATM* and *p53* influence the repair of the I-SceI breaks.

Several important technical caveats should be considered when utilizing the I-SceI system (Figure 1a). Firstly, there is usually substantial variation in the induction of recombination among several single-cell derived clones, implying that the random integration site can have considerable influence on recombination activity. One way of obtaining a representative sample of the cell population under study is to pool a large number of colonies, which contain plasmids integrated at various random sites in the genome. Secondly, in many situations, extremely large amounts of I-SceI expression vector, that is, up to 100 μg, need to be transfected for the effect to be seen. One possible explanation is that simple religation of I-SceI break ends is the dominating activity and that therefore serial DSBs have to be induced to eventually trigger an HR event. Thirdly, spontaneous HR frequencies can be substantial and thus mask any DSB-induced recombination activity. However, it can be useful to isolate a clone that exhibits a significant spontaneous HR frequency and an at least 10-fold induction after the expression of I-SceI nuclease. This would allow for parallel evaluation of spontaneous and break-induced HR, which potentially have different genetic determinants. Lastly, it is important to stress that the experiment typically spans two or more months involving several rounds of transfection and colony formation. Therefore, it is favorable to develop shorter assays that make use of recombination markers such as the green fluorescent protein (GFP), which will abrogate the need for one round of colony formation in difficult to grow cell lines [14].

Cell line and break type limitations

From the data obtained with the I-SceI repair system, arises the question whether this assay should be considered the "gold standard" to assess proficiency in homology-mediated DSB repair. This assay provides an important model system in cell lines that are characterized by rapid and unlimited growth in tissue culture, by high transfection efficiencies (eg, > 10%) and sufficient colony forming abilities (eg, > 5%). However, these requirements are only met in a selected number of cell lines including immortal lines such as Chinese hamster ovary cells or murine embryonic stem (ES) cells. It is important to recognize that immortal cell lines typically do not possess true functional wild-type p53 status, including ES cells [24, 25, 26]. However, with the loss of p53, the regulation of HR is expected to be relaxed or disturbed [20, 24]. This likely affects the functional analysis of other proteins involved in HR. In most cell lines with wild-type p53 status, successful application of colony formation assays to measure HR is difficult to accomplish because of p53's growth-inhibitory effects. Exceptions may include some tumor lines, for example, MCF-7 [20], and immortal lymphoblastoid lines [27, 28]. Typical limitations to the utilization of complex repair assays such as the I-SceI system are displayed in Table 1.

Induction of chromosomal breaks by the I-SceI endonuclease generates a 4 bp staggered cut that leaves free 3′ hydroxy overhangs, which can be directly religated. As discussed, the proficiency to repair this break type via HR correlates with the resistance to IR. However, IR creates far more complex chromosomal break ends than endonucleases, and it is therefore likely that several protein activities that participate in the repair of radiation-induced chromosomal breaks can be missed by an I-SceI type assay. The technical challenge, therefore, is to design assays that employ ends resembling radiation type damage more closely, and this could include cohesive but noncompatible cut sites, blunt ended cuts, and dephosphorylated double-stranded ends. Consequentially, such an approach would involve variably cut extrachromosomal repair substrates, as no alternative to I-SceI generated chromosomal breaks is yet available.

Plasmid assays containing extrachromosomal components

The role of ATM for extrachromosomal and integration-associated HR was investigated in our laboratory by Luo et al (Figure 1b) [18]. This assay studies intermolecular HR between two plasmids, pΔ2 and pΔ3, which contain a 5′ deletion and a 3′ deletion in the bacterial *gpt* gene, respectively. The gene arrangements are illustrated in Figure 2. The HR and restoration of the *gpt* gene following the cotransfection of the plasmids resulted in a resistance to XHATM in a colony formation assay. When the plasmids were cleaved at a distance from the gene, the HR frequency was 27-fold higher in AT fibroblasts than in normal human fibroblasts. However,

Table 1. Typical limitations to the use of I-SceI based plasmid assays for assessment of chromosomal DSB repair.

Limitations	Cell type
Limited life span	Primary MEFs Primary human fibroblasts
Poor tissue culture features (limited colony formation, growth, and transfection efficiency)	Primary MEFs (especially BRCA1/2 knock-outs) Primary human fibroblasts (especially NBS1) Many tumor lines (eg, Capan-1)
Alteration of wild-type p53 function	Immortalized MEFs Mouse ES cells SV-40 transformed human fibroblasts Most human tumor lines
Apoptosis proficiency	Lymphoblastoid lines

MEF: mouse embryonic fibroblasts.

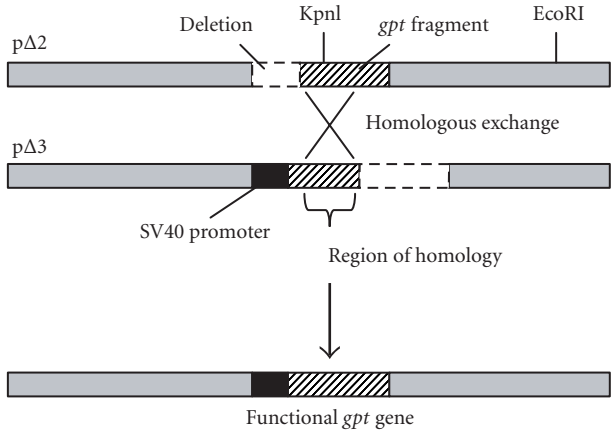

Figure 2. Illustration of integration-associated HR (corresponding to Figure 1b). Plasmid substrates pΔ2 and pΔ3 contain nonoverlapping 5′ and 3′ deletions, respectively. There is an area of shared homology of approximately 400 bp. Experiments were carried out with cleavage at a distance from the gene, that is, EcoRI, or within the gene, that is, KpnI. See [18] for details. Homology-mediated recombination and *gpt* gene restoration can occur before, during, or shortly after chromosomal plasmid integration. Cellular resistance to XHATM is selected for after 48 hours.

upon cleavage within the *gpt* gene, the frequencies differed only by 3-fold. One conclusion derived from these data was that if the DNA termini are within or close to the recombination substrate, then the high frequency of extrachromosomal recombination in AT cells is offset by mis-repair of the recombining sequences.

Figure 1c illustrates an assay modification, which utilizes stable chromosomal integration of pΔ2puro at a random site, transient transfection of pΔ3 and selection of *gpt* recombinants. Using this targeting principle, we could show that BRCA2- and BRCA1-deficient tumor cells are defective in homology-mediated recombination between a chromosomal and an extrachromosomal substrate by one order of magnitude ([29] and unpublished data from our lab). The results of this assay are consistent with other reports showing reduced homologous gene targeting in cells lacking BRCA2 or BRCA1 [16, 17].

Purely extrachromosomal HR, that is, in the absence of any integration step, can be assessed by employing episomal shuttle vectors (Figure 1d). Our data suggest that BRCA2 maintains its stimulatory effect on HR in cancer cells in this somewhat less physiologic assay setting [29]. In contrast, absence of murine p53 has no impact on regulation of HR in extrachromosomal plasmid substrates, in striking difference to the suppression of chromosomal HR [30]. Figure 1e illustrates a rapid plasmid reactivation assay which employs *CAT* or *Luciferase* reporters; however, it is unclear what the genetic determinants of this endpoint are.

Translational research in the postgenome era may ultimately involve routine characterization of repair phenotypes in primary fibroblasts or tumor cells directly taken from patients with known genetic profiles. Universally applicable plasmid assays that partially or entirely contain extrachromosomal components could provide a means to assess recombinational repair in cells that have limited growth characteristics, and to assess the repair of various break types. However, a significant problem to be addressed involves the physiological limits that extrachromosomal repair substrates possess. For example, extrachromosomal DNA is easily subjected to nucleolytic attack and is removed from chromatin regulation processes. Therefore, it becomes critical to correlate the results of extrachromosomal assays with those of I-SceI-based assays in defined model systems and to characterize the genetic determinants of extrachromosomal DSB repair. At this stage of research, it appears that purely extrachromosomal HR proceeds independently of p53, at least in murine fibroblasts [30]; thus necessitating the use of integration-linked assays for analysis of p53-dependent HR (Figures 1b, 1c, and 2). Alternatively, assays utilizing mutated SV40 genomes can provide an important alternative to plasmid systems for assessment of chromatin-associated repair mechanisms [19, 21, 23].

NONHOMOLOGOUS END-JOINING

Religation versus illegitimate rejoining

Nonhomologous end-joining of two double-stranded DNA ends does not require an undamaged partner and does not rely on extensive homologies between the recombining ends. Sometimes, NHEJ can utilize flanking microhomologies spanning 2–6 bp. Rejoining of the ends can occur after limited degradation at the termini. Furthermore, in many cases end-joining involves sequence alterations by small deletions, insertions, or inversions. Thus, the repair process itself is error-prone. It is important to realize that restriction enzyme generated DSBs can be repaired by either simple religation or illegitimate rejoining thereby destroying the original sequence. It has been hypothesized that illegitimate rejoining is more likely to occur with plasmid ends that have been generated prior to cell transfection, possibly because the ends are subject to degradation once entering the cell. In contrast, break induction in vivo such as by I-SceI would be more prone to religation. However, our data suggest that illegitimate rejoining of extrachromosomal I-SceI breaks can occur at a high frequency, though it is difficult to assess the contribution of religation because a religated break is indistinguishable to a recognition sequence that has never been cut [30]. Lin et al [13] introduced a chromosomal repair substrate into mouse L cells, which contained a *TK* gene disrupted by an I-SceI site tandem repeat. Generation of DSBs led to the removal of the intervening sequence and precise religation with reconstitution of one I-SceI site in 33–65% of all NHEJ events. This is likely to be an overestimate, since NHEJ not resulting in restoration of the *TK* open reading frame could not be selected for. In contrast to cuts from endonucleases or restriction enzymes, radiation-induced breaks can only be repaired by an illegitimate repair process.

The dilemma of selection

In contrast to HR, it is difficult to envision how NHEJ can reconstitute the function of a reporter gene, which is needed to allow the identification of repair products within a large cell population. When using a chromosomally integrated reporter gene that is inactivated by the insertion of an I-SceI recognition site, NHEJ would have to precisely remove the insert in order to reconstitute gene function. In principle, this can be facilitated by placing regions of microhomology upstream and downstream of the insert. However, such an assay would only score a fraction of NHEJ events. Previously, investigators have grown up 100 or more unselected colonies after induction of I-SceI breaks in an integrated HR substrate (Figure 3a). Physical analysis using Southern blotting and PCR on a clone by clone basis can provide a quantitative estimate of chromosomal NHEJ; however, this is an extremely laborious process. The only available assay type that uses drug selection for NHEJ events involves measuring the frequency of random stable integration of a linearized plasmid substrate (Figure 3b). Cells lacking components of the nonhomologous repair pathway, for example, DNA-PK$_{cs}$, Ku, or XRCC4, display plasmid integration frequencies that are reduced by 10-fold or more ([31] and unpublished data from our lab). However, factors other than break end-joining proficiency may influence plasmid integration, thus making this assay subject to various biases.

As with HR, NHEJ has been historically studied using extrachromosomal substrates. Several assay principles have been employed. Figure 3c illustrates a variation that we have used recently [32]. Circular plasmid molecules were linearized between the viral promoter and the *Luciferase* reporter gene prior to transfection. In linearized plasmids, the *Luciferase* gene cannot be expressed. Only after DSB rejoining with recircularization of the plasmid, the transcription of the reporter gene can proceed. We showed that p53 could enhance NHEJ of DSBs with cohesive ends by 2- to 3-fold in rodent embryonic fibroblasts, but only in the presence of an additional exogenous DNA damage signal. The data suggested that p53 was enhancing DSB rejoining specifically by increasing the ability to reanneal short complementary strands of single-stranded DNA. Using a similar assay principle, Liang and Jasin [33] observed increased degradation of DNA termini in Ku80-deficient cells. However, in cells lacking DNA-PK$_{cs}$, plasmid rejoining proceeded normally. It is therefore not clear as to which degree the pathways involved in nonhomologous repair of radiation-induced DSBs and in the rejoining of extrachromosomal plasmid ends overlap. A more specific way to study the genetic determinants of NHEJ is to utilize Rag1- and Rag2-initiated site-specific V(D)J recombination as a functional endpoint, which tests for the functional presence of the DNA-PK complex, XRCC4, and ligase IV (Figure 3d) [34]. This assay is analogous in design to the HR shuttle vector assay.

In summary, as for HR, a variety of assays are available to assess NHEJ in difficult and easy to grow murine and human cell lines. However, more study is needed to determine which genetic pathways can be studied by any particular system.

TOWARD THERAPEUTIC GAIN

For genotoxic agents used in cancer treatment, a therapeutic gain is defined by a better relation between the killing of tumor and normal cells in a patient. With regard to IR, it is important to realize that in most instances malignant cells are neither more radiosensitive nor more radioresistant than their normal counterparts. In some cases, mutations in central recombination genes within the tumor appear to confer a defect in DSB repair and consequential radiosensitivity, as suggested for BRCA2 [29]. Such a mutation may either be absent in the normal tissues, or be present as heterozygosity but not conferring any repair phenotype, thus offering therapeutic gain upon treatment with DSB-inducing agents.

It is likely that recombinational repair in malignant cells is generally altered compared to normal cells. For example, the *P*53 gene is mutated or inactivated in the majority of human tumors. Consequentially elevated HR may contribute to the increased loss of heterozygosity and chromosomal instability observed in many tumors, though the causal

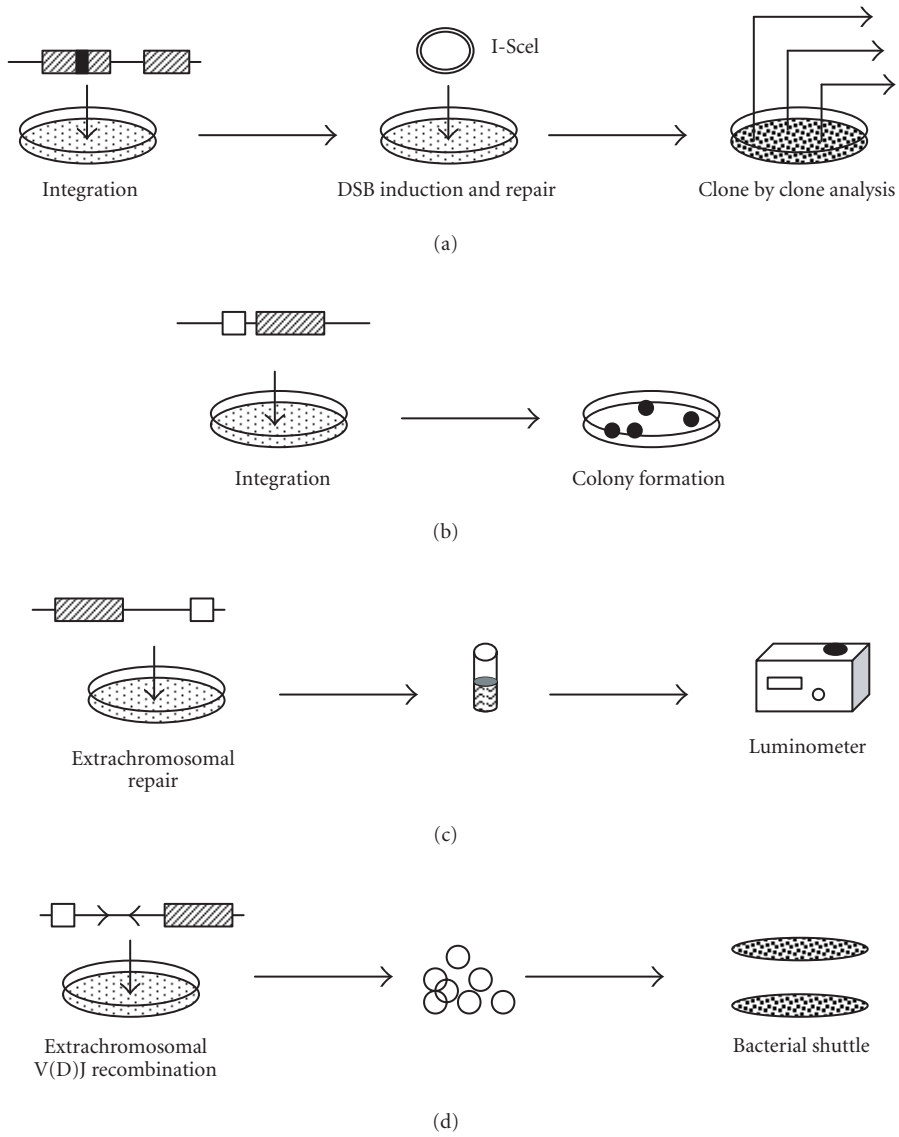

FIGURE 3. Illustration of the principles of measuring NHEJ with plasmid assays. (a) Physical analysis of chromosomal NHEJ. Analogous to Figure 1a, I-SceI breaks are generated in an integrated HR substrate, but cells are plated without selection antibiotic. Colonies are analyzed on a clone by clone basis for an NHEJ product using Southern blot and PCR. Alternatively, PCR of genomic DNA can be used for semiquantitative analysis of NHEJ [16]. (b) Random plasmid integration [29, 31]. The transfection frequency of a linearized reporter plasmid as a measure of NHEJ is scored by selecting for the intact resistance gene, for example, with puromycin. (c) Extrachromosomal rejoining assay [32]. A *Luciferase* expression plasmid is cleaved between the promoter and the reporter gene prior to the transient transfection. In the cell, *Luciferase* can only be expressed following NHEJ and re-circularization of the plasmid. Cell extracts are assayed for *Luciferase* activity. (d) Episomal V(D)J recombination [34]. Analogous to Figure 1d, recombination signal sequences flank a bacterial transcription stop signal upstream of a *CAT* reporter. The plasmid is cotransfected with Rag1/Rag2 expression vectors. The cleavage of the signal sequences followed by site-specific recombination removes the transcription stop signal, so that extracted plasmids can be assessed for *CAT* resistance in a bacterial shuttle vector assay.

relationship has yet to be proven [2]. The loss of cell cycle control in tumor cells results in a larger fraction of cells being in the S and G2 phases of the cell cycle compared to normal cells. Homology-directed DSB repair is thought to be an important pathway in these phases. This is in contrast to the G1 phase, during which NHEJ is likely to dominate because no sister chromatid is available to provide a homologous repair template. Therefore, HR may be commonly elevated and deregulated in cancer cells. This could provide a rationale basis for drug design targeting HR pathways and thereby sensitizing tumors to IR. Therapeutic gain will result especially in relation to non- or slowly proliferating normal tissues, which are largely in the G0 or G1 phase and thus repair mainly via NHEJ. A variety of functional assays including plasmid systems and other means will be needed to characterize DSB repair in cancer and normal cells and to

allow predictions of the outcome of combined treatment approaches. However, as discussed here, several technical challenges with respect to functional analysis in vivo still need to be overcome.

ACKNOWLEDGMENTS

Part of this work was supported by grants from the National Cancer Institute to S. N. Powell and by a grant from the Massachusetts Department of Public Health to F. Xia.

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Journal of Biomedicine and Biotechnology • 2:2 (2002) 94–105 • PII. S1110724302205033 • http://jbb.hindawi.com

REVIEW ARTICLE

Regulation of Repair by the 26*S* Proteasome

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Received 30 April 2002; accepted 10 May 2002

Cellular processes such as transcription and DNA repair may be regulated through diverse mechanisms, including RNA synthesis, protein synthesis, posttranslational modification and protein degradation. The 26*S* proteasome, which is responsible for degrading a broad spectrum of proteins, has been shown to interact with several nucleotide excision repair proteins, including xeroderma pigmentosum B protein (XPB), Rad4, and Rad23. Rad4 and Rad23 form a complex that binds preferentially to UV-damaged DNA. The 26*S* proteasome may regulate repair by degrading DNA repair proteins after repair is completed or, alternatively, the proteasome may act as a molecular chaperone to promote disassembly of the repair complex. In either case, the interaction between the proteasome and nucleotide excision repair depends on proteins like Rad23 that bind ubiquitin-conjugated proteins and the proteasome. While the iteration between Rad4 and Rad23 is well established, it will be interesting to determine what other proteins are regulated in a Rad23-dependent manner.

INTRODUCTION

The regulation of DNA repair is important for cell survival following exposure to DNA-damaging agents. A large array of exogenous and endogenous agents can interact with and cause DNA damage, interfering with essential cellular processes, such as transcription, DNA replication, and cell-cycle progression. Disruption of these processes can lead to cell death. Alternatively, unrepaired or mis-repaired DNA can generate mutations that lead to cellular aging, genetic defects, and carcinogenesis. One major pathway that contributes to the removal of DNA damage is the nucleotide excision repair (NER), whose biochemical mechanism has been characterized extensively. In contrast, the regulation of NER is not well understood. Regulation of NER could be accomplished through changes in RNA transcription, protein translation, protein degradation, or posttranslational modifications.

A burgeoning literature underscores the relevance of the ubiquitin(Ub)/proteasome pathway to many cellular processes. Interactions between the proteins involved in NER and the Ub-mediated protein degradation pathway have been reported for yeast and mammals. The emerging evidence that yeast repair protein Rad4, and its human counterpart XPC, might be targeted for degradation by the 26*S* proteasome is consistent with a negative role for Ub-mediated proteolysis in NER. In addition, the intact 26*S* proteasome, or its constituent parts, may serve additional roles in NER, perhaps as molecular chaperones that promote the proper folding of repair proteins or disassembly of protein complexes.

NUCLEOTIDE EXCISION REPAIR

The nucleotide excision repair (NER) pathway in eukaryotes is conserved from yeast to humans. This repair system removes many bulky chemical adducts and UV-induced photoproducts from DNA in a relatively error-free manner. Defects in nucleotide excision repair are associated with increased incidence of cancer. The identification and cloning of genes involved in NER has led to the reconstitution of repair in vitro [1, 2], using approximately 30 purified proteins [3, 4]. Nucleotide excision repair proteins have been purified from yeast cell extracts as functional subassemblies called nucleotide excision repair factors (NEFs). NEF1 contains a damage recognition protein Rad14, and a 5′-endonuclease complex (Rad1/Rad10) that binds preferentially to damaged DNA. NEF2 consists of Rad4 and Rad23, which can also bind preferentially to damaged DNA, and might play a role in recruiting other repair proteins to the sites of DNA lesions. NEF3 contains Rad2, an endonuclease that cleaves on the 3′ side of the DNA lesion, and TFIIH, an RNA polymerase II-associated transcription factor complex. NEF4 consists of Rad7 and Rad16 and, like NEF1 and NEF2, binds preferentially to damaged DNA. As in bacteria, NER in eukaryotic cells is a multistep process that recognizes damaged DNA, generates incisions upstream and downstream from the lesion, and displaces

the damaged DNA as part of an oligonucleotide (Figure 1) [2]. The excised oligonucleotide in human extracts is larger than in *E coli* because the 5′ incision is made farther from the lesion. The gap left after the removal of the lesion-containing oligonucletide is repaired by DNA polymerases δ and ε, and ligated to the flanking parental DNA by DNA-ligase. The biochemical details of DNA incision and excision, which have been extensively characterized in yeast, display remarkable mechanistic conservation with the human system [5].

NER comprises two subpathways that either target transcribed strands of class II genes, or nontranscribed sequences (that includes the genome overall). These two subpathways share many factors, though some are unique to one subpathway or the other.

GLOBAL GENOMIC REPAIR IN YEAST

The repair of nontranscribing sequences within genes occurs at about the same rate as the repair of overall genomic DNA, and requires the proteins shown in Figure 1, in addition to Rad7 and Rad16 (NEF4), which have been shown to bind UV-damaged DNA [6]. Importantly, Rad16 is a member of the Swi2/Snf2 family of DNA-dependent ATPases that are thought to remodel nucleosomes, and/or displace proteins from chromatin [7]. However, a clear-cut homolog of Rad7 or Rad16 has not been identified in mammals.

TRANSCRIPTION-COUPLED REPAIR IN YEAST

In addition to the recognition and excision of damaged DNA that occurs throughout the genome, there exists a specific mechanism that recognizes damaged DNA that is present in the transcribed strand of genes that encode mRNA [8, 9, 10, 11]. The preferential repair of the transcribed strand is absolutely dependent upon the transcription by RNA polymerase II [8, 11]. For instance, the removal of cyclobutane pyrimidine dimers (CPDs) from the transcribed strand of *RPB2* (which is transcribed by RNA polymerase II), is much more rapid than from the nontranscribed strand. When exponentially growing *rpb1-1* cells are shifted to the nonpermissive temperature, they rapidly cease mRNA synthesis, and the repair of the transcribed strand is reduced to that of the nontranscribed strand. Similarly, the transcription defect that is associated with the heat-sensitive *kin28ts* mutant (that is a component of TFIIK) is accompanied by a defect in transcription-coupled repair [12]. Preferential repair of the transcribed strand of the mammalian *DHFR* gene is also abolished by the treatment of cells with the RNA polymerase II inhibitor α-amanitin [13, 14]. Collectively, these findings provided compelling evidence that the transcription by RNA pol II contributes to the strand bias in DNA repair. Although it was previously assumed that TCR was confined to genes that are transcribed by RNA polymerase II, recent studies demonstrated that TCR is also present in the transcriptionally active fraction of ribosomal DNA (rDNA), which is transcribed by the RNA polymerase I [15].

TRANSCRIPTION REPAIR-COUPLING FACTORS IN EUKARYOTES

CSB and *CSA* are the only genes that have been reported to affect transcription-coupled repair in mammalian cells. *CSB* plays a specific role in resuming RNA synthesis following the UV irradiation [16, 17]. The yeast homolog of the CSB (Rad26) was identified, though its biochemical function is unclear [18, 19]. Rad26 and CSB are members of the Swi2/Snf2 family of DNA-dependent ATPases that are thought to remodel nucleosomes, and/or displace proteins from chromatin [7]. Indeed, CSB has been demonstrated to remodel nucleosomes in vitro [20]. A yeast *rad26* disruption mutant displayed similar rate and extent of removal of CPDs from the transcribed and nontranscribed strands of the *RPB2* gene [19], revealing a defect in transcription-coupled repair. This defect of the *rad26* mutant is consistent with the transcription coupled repair defect of cells that were derived from patients with Cockayne syndrome. However, in contrast to Cockayne cells, *rad26* mutants are not UV sensitive, perhaps due to the proficient repair of the overall genomic DNA in yeast [19]. The corresponding lack of UV sensitivity in *rad26* mutants could explain why they were not isolated in genetic screens that sought mutants that were sensitive to UV, or defective in overall genomic repair.

The biochemical steps that underlie NER have been carefully elucidated, though the mechanism that enables the repair machinery to distinguish between transcriptionally active and inactive DNA remains enigmatic. While *CSA* and *CSB* (*RAD26*) genes in mammalian and yeast cells clearly play a role in the transcription-coupled repair, their biochemical activities remain to be defined. For instance, it is not known if the CSA or CSB function as transcription-repair coupling factors. There is evidence that CSB can affect transcription elongation in vitro [21], though CSB does not colocalize with RNA pol II in vivo [22]. CSB might deliver CSA to RNA pol II that is stalled at sites of DNA damage [22].

GENETIC EVIDENCE FOR A PROTEOLYTIC ROLE IN DNA REPAIR

Several lines of genetic evidence suggest a role for ubiquitylation and protein degradation in DNA repair. Mutations in the genes encoding Rad6, Ubc13, Mms2, Ufd2, p53, Rad16, Ump1, and Rad23 render cells sensitive to UV irradiation or stressful conditions. Rad6, Ubc13, and Mms2 are required for ubiquitylation of substrate proteins, while Ufd2 and Rad23 are likely to regulate this process [5, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32]. Ump1 is a maturation factor that is required for the assembly of the catalytic subunit of the proteasome [33, 34]. Rad16 is a member of the Swi2/Snf2 family of chromatin remodeling ATPases that contains a zinc finger motif (RING), which is also present in several Ub-specific proteolytic factors [35]. The tumor suppressor protein p53 is targeted for ubiquitylation and degradation by the 26*S* proteasome [36, 37, 38]. However, DNA damage results in its stabilization, and elevated levels of p53 permit activation of

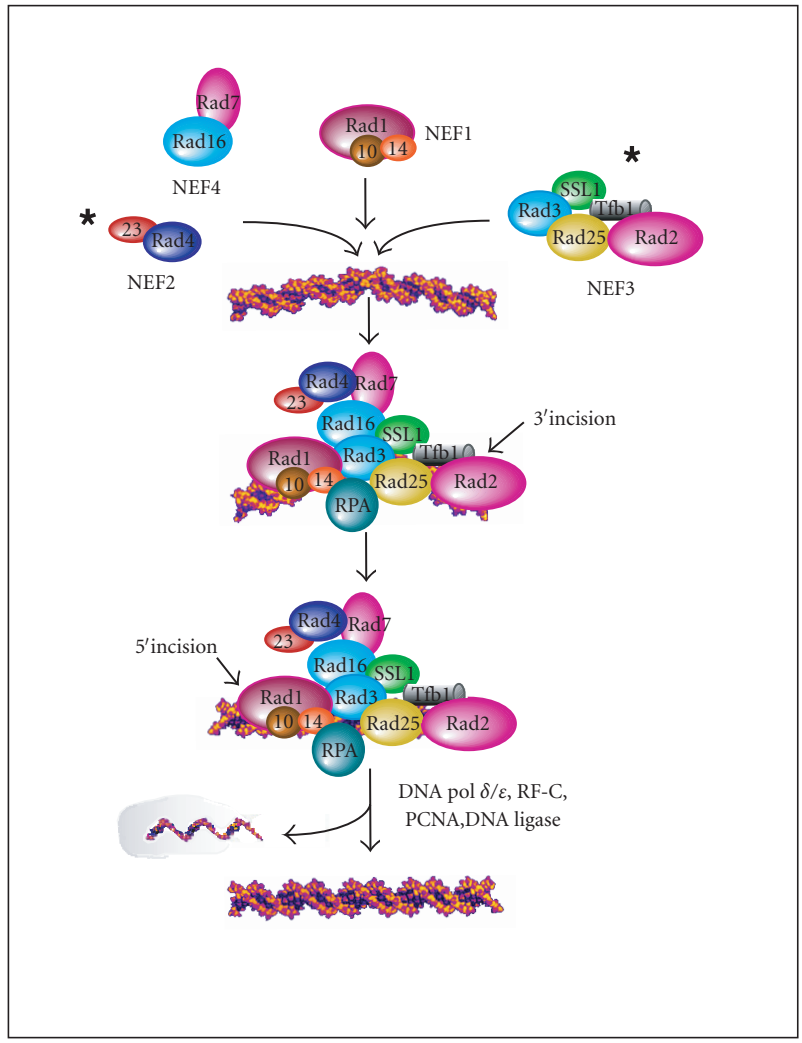

FIGURE 1. A model of yeast nucleotide excision repair. The biochemical details of the incision and size of the DNA oligonucleotide that is excised by yeast NER proteins has been determined, and is mechanistically similar to the human system [2, 5]. An asterisk indicates the presence of repair proteins that interact with the proteasome. Mutations in most genes that encode nucleotide excision repair proteins result in severe UV sensitivity. The order of repair complex assembly has been extensively studied, though the biochemical steps that follow DNA excision and gap-filling are unknown. The recycling of RNA polymerase II following exposure to a DNA-damaging agent has been suggested [76]. However, the lack of an in vivo or in vitro assay to measure the recycling, dismantling, and degradation of the repair complex, reveals significant steps in the cellular response to DNA damage that remain to be defined.

genes that contribute to enhanced survival in response to various environmental stresses. Nucleotide excision repair proteins, Rad4 and XPB, interact with the proteasome, while Rad4 can be copurified with the proteasome. Mutations in the genes encoding Rad4 and XPB cause severe UV sensitivity compared to repair-proficient cells. There is emerging evidence that Rad4, and its human counterpart (XPC), are ubiquitylated and degraded by the 26*S* proteasome (see section Stabilization of a Repair Protein in a Proteasomal Mutant below). Similarly, it is possible that the ubiquitylation and degradation of other repair proteins could also govern the efficiency of DNA repair.

The expression of several genes that encode components of the ubiquitin/proteasome pathway is induced following DNA damage, consistent with a possible role in DNA repair (see section Many Ubiquitin/Proteasome Genes Are Induced by DNA Damage below). For instance, the expression of both *RAD6* and *RAD23* is elevated after DNA damage. Additionally, the treatment of cells with methylmethane sulfonate (MMS) resulted in induced expression of several genes in the ubiquitin/proteasome pathway, consistent with the existence of a network that coordinates the regulated expression of ubiquitylation and proteolytic enzymes in response to DNA damage.

TRANSCRIPTION-COUPLED REPAIR AND UBIQUITIN-MEDIATED PROTEIN DEGRADATION

The suggestion that ubiquitylation, and perhaps protein degradation, might play a role in NER was made by Bregman

and colleagues [39]. HeLa cells and normal human fibroblasts in culture can ubiquitylate the large subunit of RNA polymerase II (RNA pol II LS) following UV irradiation or treatment with cisplatin [39]. The ubiquitylation of RNA pol II LS was absent in UV-irradiated fibroblasts from Cockayne syndrome patients. In contrast, fibroblasts derived from patients displaying symptoms of xeroderma pigmentosum, another autosomal recessive disorder associated with a DNA repair deficiency, were capable of ubiquitylating RNA pol II LS following UV irradiation [40]. These findings may indicate that the failure to regulate the stability of RNA pol II might underlie the DNA repair-specific defects of Cockayne syndrome.

It has been shown that some types of DNA lesions in the transcribed strand can arrest the movement of the transcription complex. In both normal and some *xeroderma pigmentosum* fibroblasts, the ubiquitylated form of RNA pol II LS was hyperphosphorylated, a form that is associated with the elongating transcription complex [40]. These findings lead the authors to offer a plausible role for ubiquitylation and degradation of the hyperphosphorylated form of RNA Pol II. The recognition of the stalled RNA Pol II by a ubiquitin-protein (E3) ligase could result in ubiquitylation and translocation to the proteasome for degradation. In agreement with this conjecture, Lee et al [41] demonstrated that an RNA pol II elongation complex that is stalled at a site of DNA damage can be ubiquitylated in vitro.

Yeast Rpb1 (RNA pol II LS) is ubiquitylated by the ubiquitin protein (E3) ligase, Rsp5 [42, 43]. However, the ubiquitylation of Rpb1 by Rsp5 is not required for transcription-coupled repair in yeast [44], since a strain expressing a conditional mutant of *RSP5* was proficient in transcription-coupled and nucleotide excision repair. These results suggest that the ubiquitylation of Rpb1 could occur after the transcription-coupled repair is achieved, or might even be unlinked to the repair process. However, the lack of a repair defect in *rsp5* mutant cells does not preclude a role for proteolysis in transcription-coupled or nucleotide excision repair [45], since there is evidence that the RNA pol II that is stalled at the sites of DNA damage may have several fates. While RNA pol II could be removed from damaged DNA and degraded, it is also conceivable that the Rad26 protects RNA pol II from degradation to enable TCR [46].

We propose that the regulation of NER could be achieved by controlling the abundance of one or more NER proteins, through selective degradation or protein synthesis. In agreement with this conjecture, we note that most NER genes in yeast are constitutively expressed, and only a few are expressed at higher levels following DNA damage. Therefore, an efficient DNA repair would only require the expression or stabilization of one or a few nucleotide excision repair proteins. We presume that once the repair is completed, and the nucleotide excision repair machinery is no longer required, a subset of the NER proteins would be degraded to reduce the NER activity so that the inadvertent incision of DNA structures that are generated during normal cellular transactions are avoided.

THE UBIQUITIN-MEDIATED PROTEIN DEGRADATION PATHWAY

The ubiquitin/proteasome system regulates protein stability and function and is conserved from yeast to humans, similar to the nucleotide excision repair pathway. Cellular processes that are regulated by proteolysis include apoptosis, cell cycle progression, stress responses, development, and transcriptional regulation. Defects in proteasomal function have pleiotropic effects and are implicated in lung cancer, Angelman syndrome, muscle wasting, Parkinson disease, and inflammatory response [47]. However, the ubiquitylation of a protein does not necessarily lead to the destruction of the modified protein. For instance, ubiquitylation can modify protein activity, or promote the targeting of proteins to vesicles during endocytosis [48]. The ubiquitin-mediated protein degradation pathway is depicted in Figure 2. Once the first ubiquitin is attached to a protein, specific ubiquitin chain assembly factors (E4) may promote the formation of multiubiquitin chains, which allow the target protein to be recognized and degraded by the 26*S* proteasome.

THE 26*S* PROTEASOME

The 26*S* proteasome consists of two distinct subunits, a 19*S* regulatory particle and a 20*S* catalytic core particle [49]. The 20*S* particle consists of four heptameric rings that form a barrel-shaped protein complex with the catalytic sites confined to the interior surface (Figure 3). Narrow ports at each end inhibit access to the interior. A 19*S* regulatory subunit spans each end of the 20*S* particle and restricts access to the catalytic core. Proteins appear to be de-ubiquitinated and unfolded by the 19*S* regulatory particle, before being funneled into the interior of the 20*S* catalytic core for degradation. In contrast, ubiquitin is recycled. In yeast and humans, the intact 26*S* proteasome appears to be the functional form of the proteasome [50, 51], and the association of two 19*S* subunits with each 20*S* subunit is detectable by an electron microscopy [50].

The 19*S* regulatory particle can bind multiubiquitin chains and consists of at least 17 proteins. The translocation of unfolded proteolytic substrates into the 20*S* catalytic core [49] is highly processive, as degradation intermediates are rarely detected. The six homologous AAA ATPases in the 19*S* particle are thought to unfold substrate proteins in an ATP-dependent manner, and are essential for proteasome function. Conditional mutations in these ATPases, including *cim3-1* (Sug1/Rpt6), *sug2-1* (Sug2/Rpt4), and *cim5-1* (Rpt1/Yta3) have been isolated, and their characterization has suggested that the ATPase subunits can discriminate among cellular substates, and at least one has been shown to directly recognize multiubiquitin chains. The subcellular distribution of the proteasome is a question of considerable interest, and recent reports have not clarified this issue. There is a compelling evidence that the proteasome in yeast is located primarily at the junction of the nuclear envelope and the endoplasmic reticulum [52, 53]. However, there

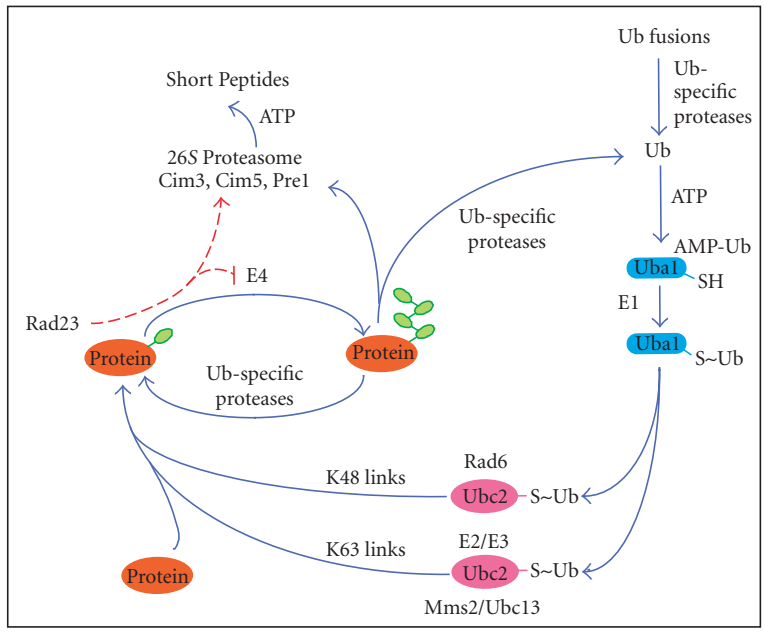

FIGURE 2. The yeast ubiquitin-mediated protein degradation pathway (adapted from [49]). The Ubiquitin (Ub) System is a multienzyme process that covalently links Ub to a wide variety of intracellular proteins. Ub is expressed as a fusion with specific ribosomal proteins or as polyubiquitin fusions. Ub-specific proteases generate free Ub that is activated by a Ub-activating (E1) enzyme, in an ATP-dependent reaction. Ub is transferred from E1 to Ub-conjugating (E2), and Ub (E3) ligase enzymes through a series of transesterification reactions, and is finally ligated to lysine residues in the target protein. This last step might require a ubiquitin-protein (E3) ligase. The specificity of the system resides in the E2 and E3 enzymes. Newly identified E4 enzymes may regulate the assembly of multiubiquitin chains on substrates.

is uncertainty as to whether the intact 26*S* proteasome contributes to all proteasomal functions, or if the 19*S* regulatory subunits can function independently of the 20*S* catalytic core [51].

The expression of a number of genes involved in NER is induced following the DNA damage, probably to hasten the removal of DNA adducts. The induced proteins are presumably degraded to restore basal levels of expression following the completion of repair. Although it is not known if nucleotide excision repair is regulated by the protein degradation, there exist several tantalizing links between these pathways. Weeda et al [54] demonstrated that the XPB subunit of human transcription initiation/repair factor IIH (TFIIH) interacts with hSUG1, the human homolog of the 19*S* regulatory subunit Cim3. XPB is the human counterpart of Rad25. Microinjection of cDNA encoding mouse SUG1 into the fibroblast nuclei led to a dramatic decrease in transcription, though there was no evidence that XPB was ubiquitiylated and degraded. Additionally, the human homologue of Cim5 (MSS1), interacts with basal transcription factors TBP, TFIIB, TFIIH, and TFIIF [55]. Weeda et al [54] speculated that the proteasome might activate proteins by processing inactive precursors. In support of this idea, it has been shown that the activation of the transcription factors NFκB and a sterol-induced regulator in yeast require proteolytic activiation of precursor proteins. Another link to proteolysis was described by Schauber et al [29] who found that Rad23 and Rad4 could be copurified with the 26*S* proteasome, though it was not clear if Rad4 was degraded. Rad23 can interact with other proteins involved in nucleotide excision repair [5, 32] as well as the 19*S* proteasome regulatory particle [28, 29]. Schauber et al [29] proposed that repair protein complex could be disassembled or degraded upon completion of repair, through a specific interaction between Rad23 and the proteasome. These studies lead the authors to suggest that Rad23 might escort proteins to the 26*S* proteasome for destruction. Alternatively, it is possible that the proteolytic activities of Rad23 are unrelated to an independent role in promoting the assembly of the nucleotide excision repair complex [56]. Both scenarios are consistent with the DNA repair defect of *rad23*Δ mutants [57].

MANY UBIQUITIN/PROTEASOME GENES ARE INDUCED BY DNA DAMAGE

Jelinsky and colleagues [58] used gene chip technology to examine the transcription profile of the *S cerevisiae* genome in response to DNA damage. Exponentially growing cultures were exposed to DNA-damaging agents and biotin-labeled cRNA was made and hybridized to an oligonucleotide array. Remarkably, almost a third of the genes showed altered expression after exposure to the DNA-damaging agent. Similarly, treatment of cells with methylmethane sulfonate (MMS) resulted in changes in the expression of ~25% of yeast genes. Some sets of genes were

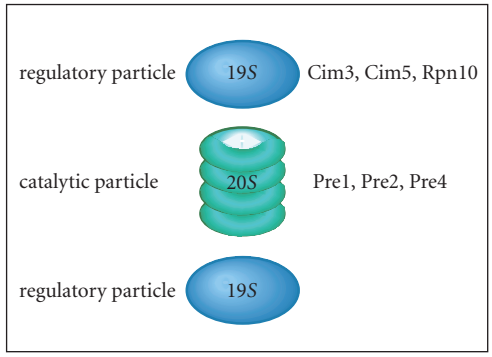

FIGURE 3. The structure of the 26*S* proteasome. Two 19*S* regulatory complexes straddle the openings to the 20*S* particle, and control access to catalytic sites within the 20*S* complex. Subunits in the base of the 19*S* regulatory particle are believed to perform a gating function that regulates the entry of unfolded proteolytic substrates into the catalytic chamber of the core particle [50]. Heat-sensitive conditional mutants in the two distinct particles of the proteasome have greatly assisted the functional characterization of the proteasome.

expressed soon after treatment, while other genes were expressed either late or transiently. Furthermore, the transcriptional response to DNA damage was cell cycle dependent. One of the genes whose expression is increased following DNA damage is *NPR1*. Npr1 protein is a putative regulator of Rsp5 (a ubiquitin protein ligase), Doa4 (a ubiquitin-specific protease), *UBC13* (a subunit of a bipartite ubiquitin-conjugating-enzyme), and subunits of the 26*S* proteasome. Sequence analysis of the promoters of many genes encoding proteolytic factors revealed putative DNA binding sites for transcription factors. Interestingly, a significant number of promoters in genes involved in Ub-mediated proteolysis contain a conserved nonameric sequence, the proteasome-associated control element (PACE), that could coordinate transcriptional activation in response to DNA damage. There is evidence that the transcription factor Rpn4 is required for stimulating transcription from PACE-containing promoters [59]. Thus, there may exist a regulatory network that coordinates the expression of ubiquitylation and proteolytic enzymes in response to DNA damage.

IN VITRO NUCLEOTIDE EXCISION REPAIR AND THE PROTEASOME

Russell et al [28] found that the addition of antibodies against the proteasome subunit Sug1 (Cim5) to a reconstituted NER reaction, inhibited repair. Furthermore, extracts prepared from yeast strains that contained mutant Sug1 or Sug2 were moderately defective in accomplishing nucleotide excision repair. Because a similar decrease in NER was not observed in yeast strains that expressed defective 20*S* subunits, it was proposed that NER does not require the proteolytic activity of the proteasome. In agreement with this result, the proteasome inhibitor lactacystin did not reduce the capacity of yeast extracts to carry out nucleotide excision repair in vitro. Furthermore, immunoprecipitation studies suggested that the 19*S* regulatory complex might function as an independent entity, distinct from the intact 26*S* proteasome that contains the 20*S* core particle. Additional support for this idea comes from identification of subunits of the 19*S* regulatory particle in the RNA pol II transcription complex [60], and the recent demonstration that the base of the 19*S* regulatory particle is recruited to promoters by Gal4 in vitro [61]. Collectively, these studies suggest that the link between NER and the proteasome might not involve proteolysis.

IN VIVO NUCLEOTIDE EXCISION REPAIR IN PROTEASOME MUTANTS

Conditional mutations in the 19*S* regulatory subunit of the 26*S* proteasome result in increased nucleotide excision repair in vivo [45], in contrast to the in vitro results with protein extracts. Repair of both the transcribed and nontranscribed strands of an RNA polymerase II-transcribed gene was increased in the absence of proteasome function, suggesting that proteolysis played a negative role in NER. In agreement with this conjecture, the over-expression of Rad4-hemagglutinin (Rad4-HA) led to increased repair of the nontranscribed strand of a reporter gene (see [45]).

STABILIZATION OF A REPAIR PROTEIN IN A PROTEASOMAL MUTANT

Pulse-chase studies revealed that Rad4-HA levels were rapidly reduced in a wild-type strain [62]. However, treatment with the UV-mimetic 4-nitro-1-quinoline (4-NQO) resulted in transient stabilization of Rad4-HA. Similarly, Rad4-HA was stabilized in yeast proteasome mutants, suggesting that the Ub/proteasome pathway mediated its degradation. To determine if Rad4-HA was ubiquitylated, we examined its levels in *cim5-1* at the nonpermissive temperature. Incubation with antiubiquitin antibodies revealed that immunoprecipitated Rad4-HA was multiubiquitylated in vivo. Over-expression of Rad23, a partner of Rad4, inhibited the multiubiquitylation of Rad4-HA, consistent with a previously described role for Rad23 in transiently stabilizing proteolytic substrates. Thus, Rad4-HA is likely to be ubiquitylated and degraded in a Rad23-dependent manner.

Similar results have been reported for mouse cells lacking both homologues of Rad23 (mHR23A and mHR23B) [63]. Homozygous loss of either mHR23A or mHR23B results in viable knockout mice, though a double mHR23A/B knockout mouse was inviable. Although the double mutant mouse was not viable, cell lines were established from the embryos and characterized for DNA repair-specific defects. In the absence of both mHR23A and B, mXPC (the mouse homolog of Rad4) was undetectable. Significantly, treating the double knockout cells with a proteasome inhibitor resulted in detectable XPC, consistent with the yeast results. These results suggest that mHR23A and B can interact with XPC and prevent its multiubiquitylated and degradation.

Cells from xeroderma pigmentosum patients of complementation group E have a defect in the repair of nontranscribed sequences. Two proteins that contribute to this

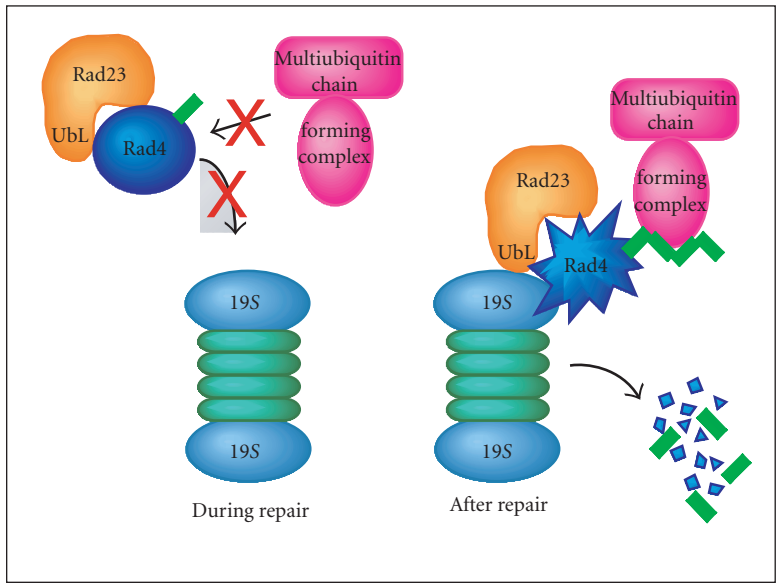

FIGURE 4. A model for regulation of repair by protein degradation. The ubiquitylation and degradation of specific repair proteins is inhibited in the presence of DNA damage. However, upon completion of repair, factors such as Rad23 might translocate repair proteins to the proteasome to promote degradation. Rad4 is a potential proteolytic target for Rad23, though there may exist other repair proteins that are degraded by the proteasome following the completion of DNA repair. It is also conceivable that negative regulators of NER, which suppress the activity of this repair pathway, are degraded following exposure to DNA-damaging agents.

defect are p48 and p125, which form a heterodimeric complex called UV-DDB that recognizes UV-damaged DNA. Intriguingly, the expression of p48 is regulated by the tumor suppressor p53, whose levels are also altered by DNA damage [64]. The p48 subunit binds to a specific E3 ubiquitin-ligase complex CUL-4A [65, 66, 67], and is subsequently ubiquitylated and degraded.

Collectively, these findings lead us to suggest that the proteins required for the nucleotide excision repair, or regulation of NER could be constitutively degraded by the 26*S* proteasome (Figure 4). However, in the absence of proteasome function, the repair proteins are expected to accumulate and increase the repair capacity of the cell. We hypothesize that following the completion of DNA repair, the repair proteins whose levels were induced are degraded by the proteasome. This mechanism of regulation could prevent improper incision of DNA structures that arise naturally during cellular processes such as transcription, replication, and recombination. This model also predicts that a failure to properly control the levels of specific repair proteins might cause deleterious effects, such as genomic instability.

THE ROLE OF RAD23 IN PROTEOLYSIS AND REPAIR

Recent observations provide support for the hypothesis that Rad23 proteins have proteolytic functions. Watkins et al [56] initially noticed that the amino-terminal domain of Rad23 (UbL) bore a striking resemblance to the sequence of ubiquitin (Ub), and intriguingly, Ub could functionally replace UbL [56]. At the time, it was proposed that Rad23 might play a role in ubiquitin/proteasome-mediated protein degradation since the only known function for Ub was its well documented effects in proteolysis. Recent studies have shown that Rad23 can bind the proteasome through its UbL domain, and can inhibit the assembly of substrate linked multi-Ub chains in a reconstituted system [68]. Additionally, a conserved motif called the ubiquitin-associated (UBA) domain has been reported to bind Ub, multi-Ub chains, and ubiquitinated cellular proteins [69, 70]. The UBA motif is present in a diverse array of regulators of signal transduction, DNA repair and proteolysis, and it seems likely that the interaction with ubiquitinated proteins provides these pathways with a previously unknown link to the proteolytic system. Other binding partners of the UBA domain have also been described, and their interaction with Rad23 can affect its DNA repair properties. Other genetic and biochemical studies are also consistent with a proteolytic role for Rad23. For instance, the loss of Rad23 in yeast cells caused stabilization of specific model substrates that was compounded by the simultaneous loss of Rpn10, a proteasome associated multi-Ub chain binding protein.

Several lines of evidence have predicted a role for the ubiquitin/proteasome system in DNA repair. Perhaps the first DNA repair protein to be directly associated with protein degradation is Rad6/Ubc2, which encodes a ubiquitin-conjugating enzyme. The *RAD6/UBC2* gene had been extensively studied and was shown to play a role in providing resistance to various types of DNA damage, meiotic recombination and sporulation, and induced mutagenesis, consistent with a role in the postreplication bypass repair. Rad6/Ubc2 is also required for retrotransposition and proper growth, and its catalytic activity as an E2 enzyme

is required for all its known functions. However, the specific targets of Rad6/Ubc2, which are related to its DNA repair specific functions, are still unknown. Similarly, two other proteins that play a role in postreplication repair are Mms2 and Ubc13, which comprise a bipartite ubiquitin-conjugating enzyme. Intriguingly, Mms2/Ubc13 assemble a unique type of multi-Ub chain that is mediated by linkages involving lysine-63 (K63) in Ub. The K63-linked multi-Ub chains are among the most abundant ubiquitinated proteins in yeast cells, and these species are not detected in cells that express the K63R Ub mutant. A subset of these bands was present at higher levels following exposure of yeast cells to UV light. Spence et al [30] reported that yeast mutants that were unable to assemble K63 multi-Ub chains were highly sensitive to DNA damage, since they were sensitive to MMS and UV light, and had reduced levels of induced-mutagenesis, consistent with the defects associated with mutations in *RAD6/UBC2*. Surprisingly, however, the K63R mutation was able to partially suppress the defects of a *rad6/ubc2* null mutant. The RAD6 epistasis pathway includes two additional members that encode potential ubiquitin protein (E3) ligases. One of these is Rad18, a single-stranded DNA-dependent ATPase that forms a high affinity complex with Rad6/Ubc2, and can potently stimulate the ubiquitin-conjugating activity of Rad6/Ubc2 on a test substrate. Additionally, Rad5 is an ATPase/helicase that displays similarity to the SNF2/SWI2 family of chromatin remodeling factors. Both Rad18 and Rad5 are putative RING-type E3 proteins that, in concert with Rad6/Ubc2, function in error-free repair. Evidence for the existence of a large complex containing both putative E3 factors (Rad18, Rad5), as well as both E2 enzymes (Rad6/Ubc2, Mms2/Ubc13), suggests that distinct types of multi-Ub chains could be assembled on DNA repair specific targets. Rad6/Ubc2 has been shown to assemble K48 chains, which are recognized by the 26*S* proteasome to promote degradation of substrates. In contrast, the K63 chains that are assembled by Ubc13/Mms2 do not appear susceptible to degradation, raising the possibility that K63 multi-Ub chains might compete with K48 chains to modulate the in vivo stability of DNA repair proteins, possibly in response to DNA damage. A clear precedence for this type of regulation is evident by the competition between a ubiquitin-like protein (SUMO-1) and Ub for ligation to physiological substrates. For instance, attachment of SUMO-1 results in the stabilization of IκBα, while conjugation to Ub results in its degradation by the proteasome. Similar findings have been reported for Mdm2, an E3 protein that regulates the stability of p53 in response to DNA damage.

Rad23 and its counterparts in humans (hHR23A and hHR23B) interact with many other proteins, including 3-methyladenine-DNA glycosylase (MPG), Png1, ataxin-3, and ubiquitin. The interaction with MPG is of particular interest because the hHR23/MPG complex binds alkylated DNA with high affinity. The C-terminal 68 amino acids of hHR23B that interact with MPG differ from the residues that bind XPC. In yeast, a protein involved in deglycosylation of misfolded proteins, Png1, also interacts with the C-terminus of Rad23. Interestingly, Png1 is a peptide-N-glycanase (PNGase) that shares a common transglutaminase fold with Rad4, suggesting that these proteins may have evolved from a common ancestoral PNGases [71]. Interestingly, the interaction between Png1 and the C-terminal UBA domain of Rad23 prevents the interaction with Rad4, though Rad4 binds a distinct region in Rad23. Because Png1 and Rad4 compete for interaction with Rad23, it is conceivable that the repair capacity of the cell is influenced by the availability of Rad23. In agreement with this idea, it was found that over-expression of Png1 prevented Rad23/Rad4 association, and was accompanied by severe sensitivity to UV light. A plausible interpretation of these results is that Rad4 is constitutively degraded in the absence of damaged DNA, and it is specifically stabilized in a Rad23/Rad4 in the presence of damaged DNA. This complex (NEF2) might then be competent for promoting the assembly of other repair proteins at the sites of lesions.

THE 26*S* PROTEASOME NEGATIVELY REGULATES DNA REPAIR

We speculate that DNA damage results in increased levels of specific repair proteins. However, the successful completion of DNA repair may be followed by the controlled degradation of repair proteins by the proteasome. The maintenance of low levels of specific repair factors could serve to carefully regulate the activity of the repair proteins, to prevent improper activities on DNA structures that occur naturally during DNA replication, recombination, and transcription. As described above in Stabilization of a Repair protein in a Proteasomal Mutant, Rad4 appears to be a substrate of the 26*S* proteasome, and its stability is likely to be controlled by Rad23. In agreement with this conjecture, we overexpressed Rad4 in wild-type cells and observed increased repair in both strands of the *RPB2* gene. These findings are consistent with the notion that specific regulatory proteins are degraded in the absence of DNA damage, but stabilized in the presence of lesions to promote repair. Rad4 may be representative of this class of proteins, and its elevated levels following DNA damage could increase transcription-coupled and genomic nucleotide excision repair.

ADDITIONAL RESPONSES TO DNA DAMAGE INVOLVING THE PROTEASOME

Not all cellular responses to DNA damage are directly related to the process of repair, since other consequences involving cell cycle arrest (checkpoint) and recovery, also involve proteolysis. Indeed, Rad23 is known to participate in a G2/M-phase transition during the cell cycle, and has been shown to control the in vivo levels of Pds1, an important regulator of cell cycle progression and DNA damage response. Furthermore, the expression of many proteins involved in transcriptional regulation is induced, and proteins are activated or stabilized in response to DNA damage.

For instance, the levels of the transcription factor Gcn4, in response to UV light and amino acid deprivation, are controlled by proteolysis and by posttranscriptional mechanisms. Gcn4 is a bZip protein of the AP-1 family that includes AP-1 and c-Jun. The protein kinase Gcn2 is essential for controlling Gcn4 protein levels in response to amino acid starvation. Gcn4 is rapidly turned over in cells growing in nutrient rich media. Gcn4 is ubiquitylated by the ubiquitin-conjugating enzymes Rad6 and Cdc34/SCF^{Cdc34} [72, 73], and degraded by the 26*S* proteasome. As expected, the degradation of Gcn4 is reduced in *rad6*Δ and *cdc34ts* mutants, and in a *cim5-1* proteasome mutant [72, 73]. Exposing cells to UV radiation or shifting glucose-conditioned cells to glucose-deficient medium results in the stabilization of Gcn4, and increased transcription from Gcn4-dependent genes [74, 75]. These results point to proteolytic effects that are manifested by DNA damage-inducing conditions, that may be quite unrelated to the enzymology of DNA repair itself.

CONCLUSIONS

Ubiquitin has been shown to participate in a variety of biochemical activities in addition to proteolysis. The ligation of mono-Ub to histone H2A and H2B has been known for a long time, though the significance of this modification is unknown. Recent studies have shown that the monoubiquitylation of numerous cell surface receptors promotes internalization and accurate localization to the vacuole or lysosome for degradation. While it was long presumed that a substrate-linked multiubiquitin chain was the singular feature that distinguished proteolytic from nonproteolytic substrates, it is now quite clear that the nature of the Ub::Ub linkage within the chain defines the fate of the targeted protein. These findings make clear that ubiquitin conjugation could have diverse biochemical effects. Consequently, the effect of the Ub/proteasome pathway in nucleotide excision repair will have to be assessed on a case-by-case basis, since it could promote the degradation of some repair factors, while altering the activity of others in a nonproteolytic manner. If Rad23-like proteins are deficient or absent in a cell, then nondegraded substrate proteins whose activity is normally regulated by mono- and di-ubiquitylation may get multiubiquitylated and subsequently degraded by the 26*S* proteasome. Even in the case of Rad23, for which proteolytic effects have been clearly ascribed, a nonproteolytic role involving Ub and the proteasome (or its subunits) in protein folding and/or disassembly of protein complexes has to be considered.

ACKNOWLEDGMENTS

The authors thank their colleagues, Lori Lommel and Mike Hampsey, for helpful comments and conversations. This work was supported by a grant from the New Jersey State Commission on Cancer Research (02-1083-CCR-SO) (KSS) and the National Institutes of Health (1R01 CA83875) (KM).

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Journal of Biomedicine and Biotechnology • 2:2 (2002) 106–113 • PII. S1110724302204040 • http://jbb.hindawi.com

RESEARCH ARTICLE

XRCC2 Is Required for the Formation of Rad51 Foci Induced by Ionizing Radiation and DNA Cross-Linking Agent Mitomycin C

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Received 28 February 2002; accepted 28 April 2002

XRCC2 protein shares weak amino acid sequence similarity with Rad51, which is a central player in homologous recombinational repair (HRR). Rad51 proteins assemble at the sites of HRR and form visible nuclear foci in response to DNA damage. *Xrcc2* hamster mutant irs1 cells are incapable of forming Rad51 foci after ionizing irradiation or DNA cross-linking agent mitomycin C treatment, though the Rad51 protein level is normal in the mutant. The defect can be corrected in an *XRCC2* transformant. Time course study showed that the irs1 cells primarily lacked the early response (2 hours after irradiation) to form small Rad51 foci (type 1) and later response (8 hours after irradiation) to form large foci (type 2). These results suggested that XRCC2 is essential for the assembly of the DNA damage-induced Rad51 foci and that XRCC2 may play an important role in the early stage of HRR.

INTRODUCTION

Xrcc2 mutant irs1 cell line is hypersensitive to various DNA damaging agents such as ionizing radiation, UV, alkylating agents, and cross-linking agents [6, 19]. Irs1 cells also show elevated levels of spontaneous or DNA damage-induced chromosomal aberrations and chromosomal rearrangement [9, 21, 45]. The human *XRCC2* gene was isolated by a functional complementation in irs1 cells, and the predicted amino acid sequence of XRCC2 protein revealed a similarity to Rad51 [7, 21]. XRCC2 belongs to a family of Rad51 paralogs, which includes XRCC2, XRCC3, Rad51B (HsRec2 or Rad51L1), Rad51C, and Rad51D (Rad51L3). All of the Rad51 paralogs share marginal sequence similarity (20–30%) with Rad51 and are present specifically in vertebrates. *XRCC3* is also isolated by functional complementation in *xrcc3* hamster mutant irs1SF [21, 43] whose phenotype is markedly similar to that of irs1 [11]. Rad51B, C, and D are identified in the database by searching for protein sequences homologous to Rad51 (reviewed in [44]). Using a recombination reporter system, it has been demonstrated that repair of site-specific DNA double strand break (DSB) mediated by homologous recombination is dramatically (100-fold) reduced in irs1 cells compared to the wild type [17]. Irs1 cells are also deficient in repair of DSBs that are induced as intermediate products in repair of DNA cross-linking damage [10]. These lines of evidence suggest that XRCC2 plays an important role in homologous recombinational repair (HRR) of DSB. However, the mechanism underlying its function is still not clear.

DNA double strand breaks are highly genotoxic lesions that can lead to chromosomal instability and mutagenesis if they are not repaired accurately. In mammalian cells, DSBs are repaired by two major pathways, the error-prone nonhomologous end-joining and the error-free homologous recombination. It is known that in yeast *S cerevisiae*, the proteins in Rad52 epistasis group (*RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, RDH54/TID1, MRE11, and XRS2*) are responsible for repair of DSBs occurred in meiosis or induced by ionizing radiation. Among these proteins, Rad51 plays a central role in HRR. Rad51 is a homolog of *E coli* RecA recombinase and its structure and function are highly conserved in mammals. Biochemical studies showed that Rad51 proteins form nucleoprotein filaments on single-stranded DNA in the presynaptic stage of HRR and mediate homologous pairing and strand exchange between single-stranded DNA and homologous double-stranded DNA [1, 14, 35]. Rad51 interacts with Rad52 and Rad54, which both promote the strand paring and exchange by Rad51 (reviewed in [38]). The yeast Rad55 and Rad57 proteins share remote sequence similarity to Rad51 and form a heterodimer that stimulates the activity of Rad51 [36]. In yeast and eukaryotic cells, Rad51 proteins form discrete nuclear foci following the induction of DSBs in meiosis or in mitotic cells treated with ionizing radiation or other DNA damaging agents [3, 15]. The biological significance of Rad51 foci is emphasized by the finding that many of the proteins involved in HRR, such as RPA (replication protein A), Rad52, and Rad54, are not only interact with Rad51, but also colocalize with Rad51 foci [23, 32, 33, 41]. In fact, Rad52, Rad54, and Rad55-Rad57 are required for the cellular response of Rad51 focus formation

[13, 41]. The breast cancer suppressor proteins, BRCA1 and BRCA2, directly or indirectly interact with Rad51 [8, 31]. These proteins are also required for Rad51 focus formation [2, 47] and their DNA damage-induced foci colocalize with Rad51 foci [8, 31]. Moreover, recent studies have shown that all of the Rad51 paralogs are essential for the formation of Rad51 foci [4, 29, 39, 40].

In this paper, I report the finding that Rad51 focus formation is defective in *xrcc2* hamster mutant irs1 cells after treatment with ionizing radiation and cross-linking agent mitomycin C (MMC). The results suggest that XRCC2 plays a role as a mediator to promote the activity of Rad51 in homologous recombinational repair.

MATERIALS AND METHODS

Cell lines

The human HeLa I and Chinese hamster cell lines V79, its derived mutant irs1, and the *XRCC2* transformants of irs1 (pDXR2 and GT621) were cultured in monolayers in α-MEM medium supplemented with 10% fetal bovine serum and antibiotics as described in [21]. The cells were grown at 37°C in a humidified 7% CO_2 atmosphere.

γ-irradiation and MMC treatment

Cells were seeded in chamber slides and grown at 37°C overnight. Cells were then irradiated with ^{137}Cs γ-rays at room temperature for 8 Gy at 1.83 Gy/min. After irradiation, cells were taken back to 37°C immediately and fixed after incubation for various period of time (30 minutes to 24 hours). For MMC treatment, cells were incubated with MMC at various concentrations (0–200 nM) in the chamber for 16 hours before being fixed.

Immunostaining

The cells grown on the chamber slides were washed 3 times with phosphate-buffered saline (PBS) and fixed with 1% paraformadehyde in PBS for 15 minutes at room temperature. Cells were permeabalized with methanol–aceton (1 : 1) on ice for 1 minute. Slides were blocked with 1% bovine serum albumin (BSA) for 1 hour, incubated with rabbit antimouse Rad51 antibody (α-MmRad51) in 1% BSA for 1 hour, and incubated with fluorescein isothiocyanate (FITC)-conjugated antirabbit IgG (Amersham, Piscataway, NJ, USA) for 1 hour. Slides were extensively washed with PBS following each incubation. Cover slices were mounted onto the slides with Vectashield mounting medium (Vector laboratories Inc, Burlingame, Calif, USA) containing DAPI (0.1 g/mL). Immunostained slides were examined under a fluorescent microscope using a 1000 × Ziess objective, and digital images were taken and recorded using software Pathvysion (Applied Imaging, San Jose, Calif, USA). At least 200 nuclei were scored and a threshold of 5 foci/cell was used.

RESULTS AND DISCUSSION

The hamster irs1 mutant cell line is effectively XRCC2 null due to an early frameshift truncation mutation [21]. Expression of human *XRCC2* partially or fully corrected the hypersensitivity of irs1 to ionizing radiation, mitomycin C, and cisplatin [21]. Since XRCC2 protein shares low level of sequence similarity to Rad51 [7, 21] and is involved in DSB repair [17], it is suggested that XRCC2 may act as a cofactor of Rad51. However, the function of XRCC2 in Rad51-mediated HRR is still unknown. To investigate the functional link of XRCC2 with Rad51, the formation of Rad51 foci is examined in *xrcc2* hamster mutant irs1. Cells were irradiated with 8 Gy γ-rays and incubated at 37°C for 2 hours, and then were fixed and immunostained with MmRad51 antibody. In wild-type V79 cells, Rad51 foci were readily detected in nuclei 2 hours after irradiation (Figure 1a). In contrast, the response of forming Rad51 foci is diminished in irradiated irs1 cells (Figure 1a). Rad51 foci are restored in an *XRCC2* genomic DNA (phage artificial chromosome (PAC) clone) transformant GT621 to the wild-type level, but are not fully restored in an *XRCC2* cDNA transformant pDXR2 (see Figure 1b). A previous study has shown that these transformants exhibit different degrees of correction for cell survival after ionizing irradiation and MMC treatment [21]. The survival of GT621 was rescued to a wild-type level after γ-irradiation and MMC treatment, while pDXR2 showed partial correction for MMC and little correction for γ-ray irradiation. The partial correction in pDXR2 cells may be resulted from an abnormal level of XRCC2 expression [21].

The frequencies of Rad51 focus-positive cells before and after irradiation are shown in Figure 1b. Before irradiation, the percentage of cells containing Rad51 foci shows no difference between V79 and irs1 cells, though the ratio (< 2%) is much lower than that in HeLa I cells (12.6%) (Figure 1b). Since the Rad51 foci seen in unirradiated cells are primarily formed in the S phase [42], this result suggests that the S phase Rad51 foci formation may not be severely affected in irs1 mutant. Two hours after irradiation, the percentage of cells containing the foci increased markedly in V79 cells (59.0%), as well as in HeLa I cells (50.7%). In addition, the number of Rad51 nuclear foci per cell is also increased in V79 and HeLa I cells after ionizing irradiation (data not shown). In contrast, irs1 cells showed no such response and the number of cells containing Rad51 foci showed little increase (Figure 1b). This result is consistent with the data reported recently, which also showed that the irs1 lacked the capability to form Rad51 foci after irradiation [29]. In GT621 cells, Rad51 focus formation was fully restored (70.9%) after irradiation (Figure 1b). However, only a slight increase of Rad51 foci was found in pDXR2 cells (14.3%) (Figure 1b). These results suggest that the response of Rad51 focus formation to DNA damage correlates well with the cellular sensitivity to DNA damaging agents.

Irs1 cells are extremely sensitive to DNA cross-linking agents, such as MMC, cisplatin, and nitrogen mustard. The increased killing of irs1 by MMC is partially corrected in pDXR2 and fully corrected in GT621 [21]. To examine the Rad51 focus formation, the cells were incubated at 37°C at various concentrations of MMC for 16 hours. In HeLa I cells, the Rad51 focus-positive cells increase after treatment with MMC in a dose-dependent manner and reached to a plateau

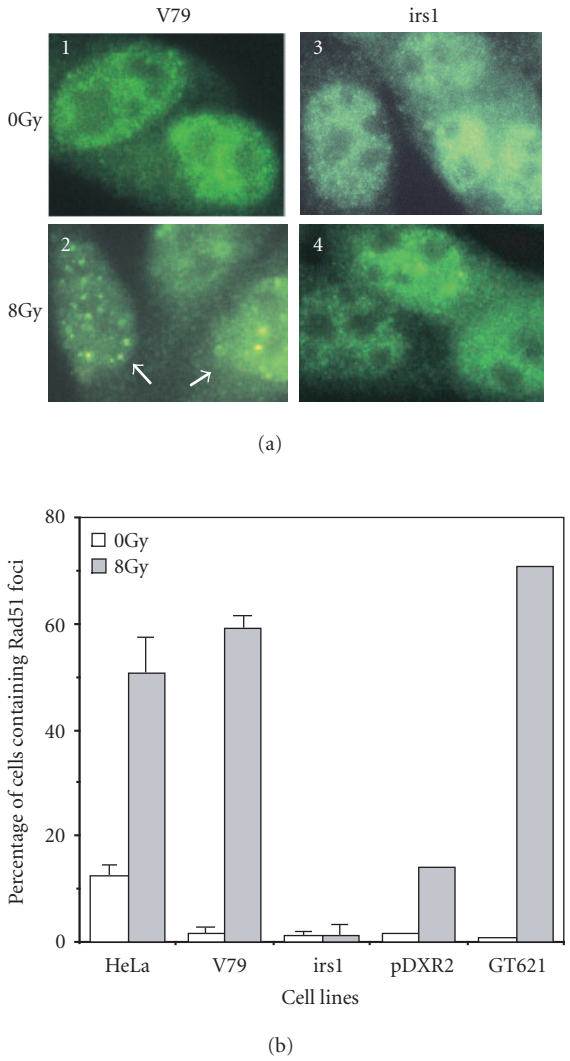


FIGURE 1. Rad51 focus formation induced by γ-ray irradiation. (a) Immunostaining with mouse α-Rad51 antibody of wild-type V79 and *xrcc2* mutant irs1 nuclei 2 hours after 8 Gy γ-irradiation. The nuclei containing Rad51 foci are indicated with arrows. (b) Percentage of cells containing Rad51 foci before and after γ-ray irradiation in HeLa, V79, irs1, *XRCC2* cDNA transformant pDXR2, and genomic transformant GT621. At least 200 nuclei for each sample were scored in each experiment and a threshold of 5 foci/cell was used. Error bars indicate the standard deviations of the average values from two independent experiments. One experiment was done for pDXR2 and GT621.

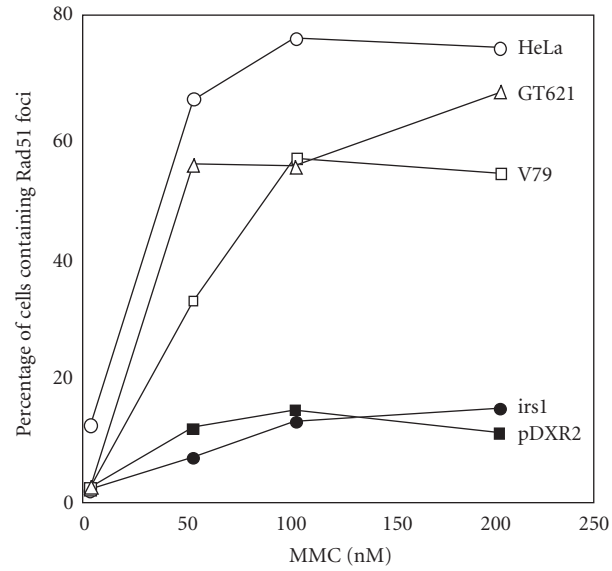


FIGURE 2. Induction of Rad51 nuclear foci by MMC treatment. HeLa, V79, irs1, pDXR2, and GT621 cells were incubated with MMC at various concentrations at 37°C for 16 hours.

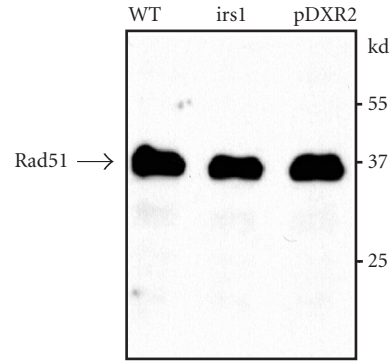
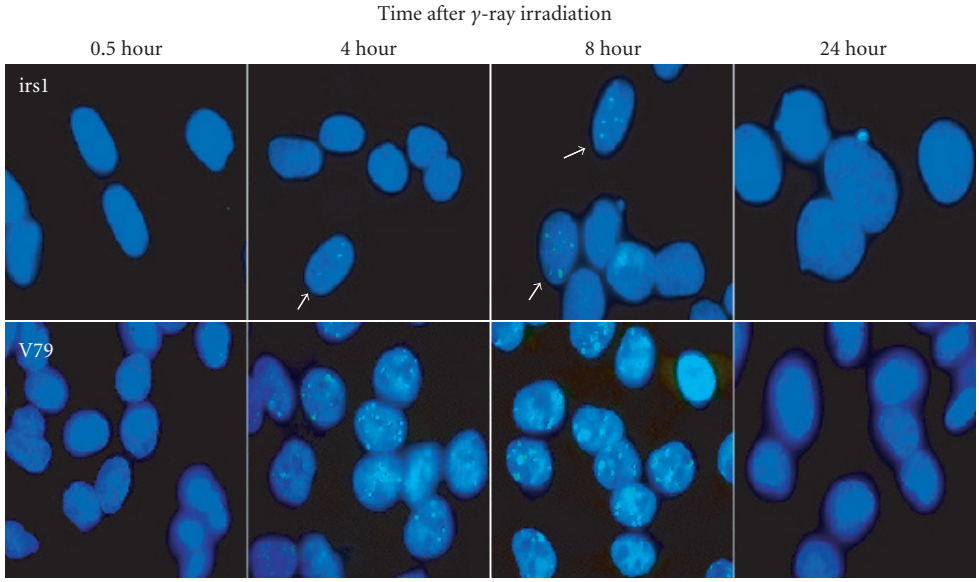


FIGURE 3. Western blot with α-MmRad51 antibody. Cell extracts (40 μg) of V79, irs1, or pDXR2 was loaded and proteins were seperated on 10% SDS-PAGE gel. The hamster Rad51 (37 kd) is indicated.

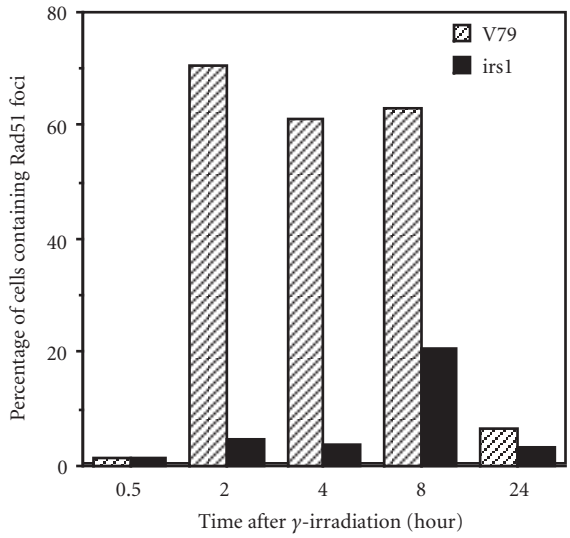
at 100 nM (Figure 2). The foci induced by MMC appeared to be smaller and less intensive than the foci induced by γ-rays. The number of cells containing Rad51 foci also increased markedly in V79 and GT621 cells after MMC treatment (Figure 2). However, the positive cells are greatly reduced in irs1 and pDXR2 cells at all dose points tested, compared to the wild type (Figure 2), though the Rad51 focus-positive cells increased slightly after continuous exposure to MMC (Figure 2).

To rule out the possibility that the diminished Rad51 focus formation in irs1 cells is due to reduced Rad51 protein level, Western blotting with α-MmRad51 antibody was performed to determine the Rad51 protein in V79, irs1, and pDXR2. Hamster Rad51 was readily detected in all cell lines and the expression levels showed no difference among V79, irs1, and pDXR2 cells (Figure 3). In addition, several studies showed that the Rad51 protein expression is not induced by ionizing radiation [4, 47], so the Rad51 level in irradiated cells should remain unchanged. This result suggests that the defect of Rad51 focus formation in irs1 is not caused by reduced Rad51 protein level, but may lie in the redistribution or assembly of Rad51 after the DNA damage. Rad51 protein level appeared to be normal in the absence of XRCC2, indicating that XRCC2 may not influence the stability of Rad51.

A recent study showed that Rad51 foci are induced by ionizing radiation during the S phase but not in G1 [12]. To ascertain whether the Rad51 focus formation is delayed in



(a)



(b)

FIGURE 4. Time course of Rad51 focus formation. (a) Combined image of FITC (Rad51) and DAPI (nuclei) staining at 0.5, 4, 8, and 24 hours after irradiation. Irs1 (top panels) and V79 (bottom panels) cells were irradiated with 8 Gy γ-rays and incubated at 37°C for various times. The irs1 nuclei contain the small foci (type I foci) are indicated with arrows. (b) Percentage of cells containing Rad51 foci at the time points after irradiation.

irs1 cells because of ionizing radiation-caused or mutagen-caused cell cycle delay, the time course of Rad51 focus formation was examined in V79 and irs1 cells. Cells were irradiated with 8 Gy γ-rays and then incubated at 37°C for various times. Very few foci was seen in V79 or irs1 cells 30 minutes after 8 Gy irradiation (Figure 4). In V79 cells, most of the cells containing the foci appeared at 2 hours and disappeared at 24 hours after irradiation (Figures 4a and 4b). The formation of the foci is significantly reduced in irs1 at all time points compared to the wild-type V79 cells (Figures 4a and 4b). However, more irs1 cells showed Rad51 foci at 8 hours compared to irs1 cells harvested at other time points. It is noticed that the foci appeared in irs1 cells 8 hours after irradiation are exclusively small foci (Figure 4a). In general, the Rad51 foci could be viewed as two types in terms of the size of the foci, the small foci (type I) and the large foci (type II) (Figure 5a). In V79 cells 2 hours after irradiation, the majority of cells contains only type I foci, while cells containing

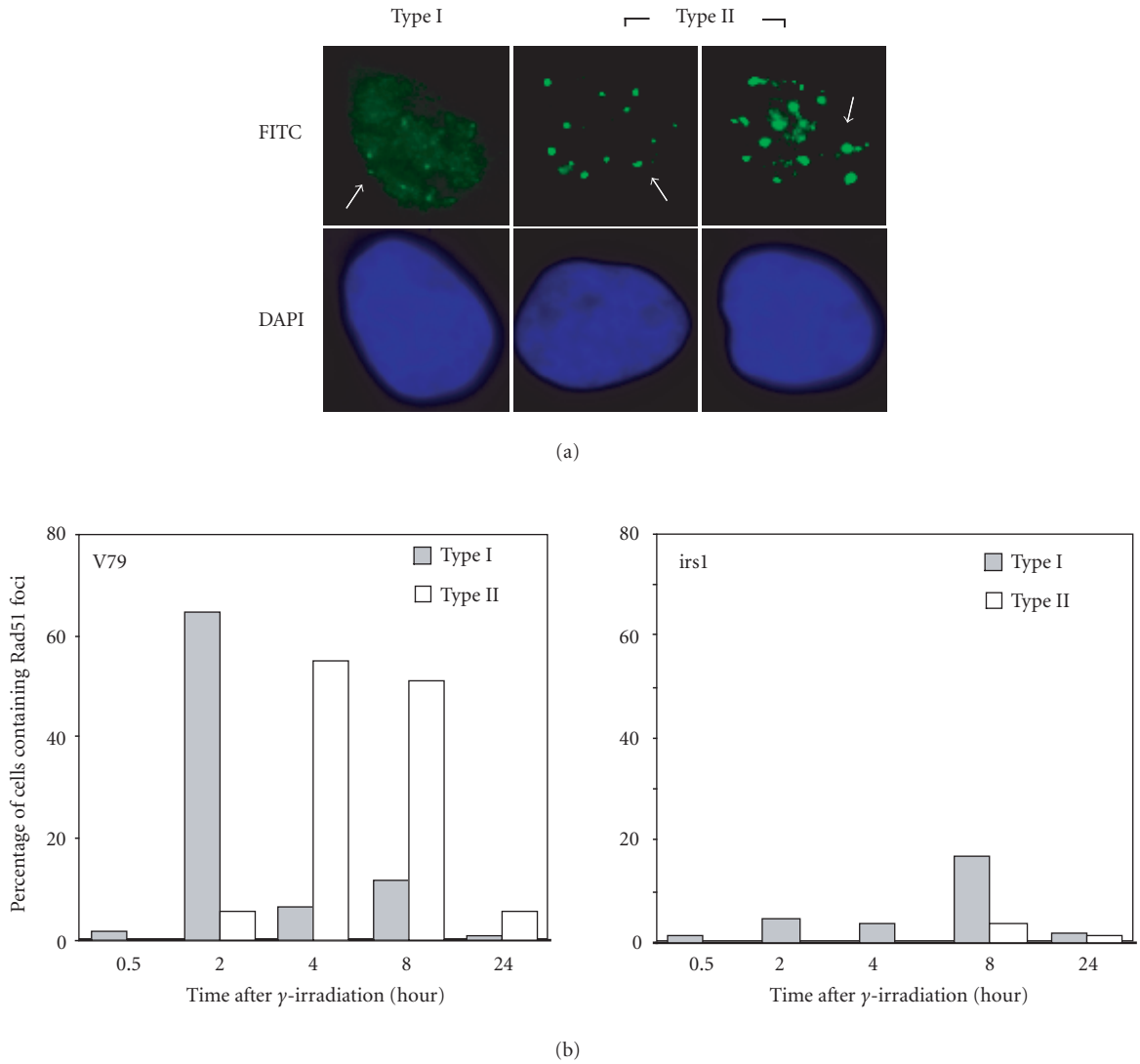


Figure 5. (a) Hamster nuclei containing type I and type II Rad51 foci (top panels). The correspondent nuclei stained with DAPI are shown in bottom. (b) Percentage of cells containing type I or type II foci in V79 (left) and irs1 (right) cells irradiated with 8 Gy γ-rays and incubated at 37°C for various times.

type II foci (> 5 type II foci/cell) became predominant at 4 and 8 hours (Figure 5b). At 8 hours, the fraction of irs1 cells containing type I (16.8%) is comparable to that of V79 cells containing only type I foci (11.8%). But the fraction of cells containing type II foci and the total number of cells containing both types of foci are significantly lower in irs1 cells than those in the wild type (Figure 5b). It should be mentioned that about 10–15% MMC-treated irs1 cells also contain type I foci after continued exposure to MMC (Figure 2). It seems that the signal for the Rad51 focus formation is normal but the kinetics for the assembly of the foci is much slower in the mutant. Taken together, these results suggest that XRCC2 is an essential factor in the assembly of Rad51.

The assembly of Rad51 proteins at the HRR sites in the presynaptic phase is a critical step for initiating homologous pairing and strand exchange. This process is mediated by a number of accessory factors of Rad51. Following the induction of DSB, the processing of the broken ends by exonucleases results in long tracks of single-stranded DNA (ssDNA) tails. Rad51 polymerizes onto ssDNA to form helical nucleoprotein filament, which is capable of conducting homologous pairing and strand exchange [37]. The biochemical study showed that DNA with single-stranded tails is the preferred substrate for Rad51 in the reaction of homologous pairing [26]. The RPA, which specifically binds to ssDNA, assembles at earlier time than Rad51 to form nuclear foci in irradiated yeast cells [12]. Binding of RPA onto ssDNA promotes the assembly of Rad51, probably by removing the secondary structure in the ssDNA [34]. However, excess RPA competes with Rad51 for binding on ssDNA and suppresses the assembly of Rad51 filament [36]. In an in vitro system, the strand exchange reaction of Rad51 is compromised if RPA is present before the nucleation of Rad51 onto ssDNA, but the reaction proceeds efficiently if RPA is added

after Rad51 binding to ssDNA [37]. The inhibitory effect of RPA can be overcome by the addition of Rad52 or Rad55-Rad57 [28, 37]. Rad54 specifically interacts with established Rad51 nucleoprotein filaments in the synaptic phase to promote homology search on the duplex DNA and heteroduplex DNA formation [33].

The function of XRCC2 and other Rad51 paralogs in the assembly of Rad51-ssDNA is not known at present. Several studies have recently shown that XRCC2 is involved in a multiprotein complex of Rad51 paralogs. Two distinct Rad51 paralog complexes have been identified in human cell extracts, one containing XRCC2, Rad51B, C, and D, and another containing Rad51C and XRCC3 [22, 24, 27, 46]. XRCC2 directly interacts with Rad51D as suggested by a yeast two-hybrid study [30]. Purified Rad51B-C-D-XRCC2 and XRCC3-Rad51C complexes preferentially bind to single-stranded, rather than double-stranded, DNA [20, 24, 25]. Significantly, the XRCC3-Rad51C complex forms protein-DNA networks in vitro [24]. These Rad51 paralog complexes also show low level of ssDNA dependent ATPase activities [20, 24, 25]. Interestingly, Rad51D [5] or Rad51C [24] alone also possesses an ssDNA-binding and an ATPase activity. The specificity of binding to single-stranded DNA would be consistent with a role of the Rad51 paralogs in facilitating the formation of Rad51 nucleoprotein filaments. One report suggested that the XRCC3-Rad51C heterodimer has homologous paring activity as determined by the D-loop formation between single-stranded and double-stranded oligonucleotides [20]. No interaction between Rad51B-C-D-XRCC2 and Rad51 is observed in cell extracts or in the reaction with purified proteins [22, 25, 27, 46]. It is suggested by a yeast two-hybrid study that Rad51 binds to XRCC3 [21]. Immunoprecipitation experiments showed that Rad51 coprecipitated with Rad51C-XRCC3 complex in HeLa S3 cell extracts [21, 22]. However, reports from other labs did not show the interaction [25, 27, 46]. Interestingly, Rad55 interacts with Rad51 in a yeast two-hybrid system [16, 18] and purified Rad55 binds to Rad51 in vitro [38], but Rad55-Rad57 is not found to coprecipitate Rad51 in cell extracts [38]. Besides, immunoprecipitation study demonstrated that neither Rad51B-C-D-XRCC2 nor Rad51C-XRCC3 interacts with RPA in human cell extracts (Liu N, unpublished data).

Vertebrate Rad51 paralogs may act as the Rad55-Rad57 complex in terms of promoting the activity of Rad51 at the early stage of HRR, but the function of vertebrate Rad51 paralogs may be diverged from their yeast counterparts. It is not known why there are two Rad51 paralog complexes in vertebrates and how they exert the function together. The Rad51 paralog complexes seem to possess a similar function but neither of them is dispensable, since the disruption of each of the Rad51 paralogs in chicken cells resulted in almost identical phenotypes [39, 40]. Moreover, Rad55-Rad57 is only required for Rad51 focus formation in meiotic cells, but not required in mitotic yeast cells [12, 13], while all of the Rad51 paralogs are necessary for the mitotic Rad51 focus formation. Biochemical studies are currently undertaken to better understand the mediator activity of the Rad51 paralogs in HRR and the co-operation of Rad51 paralogs with other mediators, such as Rad52, Rad54, BRCA1, and BRCA2.

ACKNOWLEDGMENTS

I thank Dr Larry Thompson for his encouragement and support during the study. I also thank Dr Randy Legerski for kindly supplying the mouse Rad51 antibody. This work was supported by Grant CA84407-01 from the National Cancer Institute and was performed under the auspices of the US Department of Energy by Lawrence Livermore National Laboratory under contract No W-7405-ENG-48.

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