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Structure and Functions of the Major Human AP Endonuclease HAP1/Ref-1

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1. INTRODUCTION

Apurinic/Apyrimidinic (AP) sites arise spontaneously in DNA even at neutral pH owing to the inherent lability of the N-glycosyl bond. This lability is a consequence of the absence of the sugar 2′ oxygen in DNA compared to RNA. It has been calculated that up to 10,000 bases (primarily purines) are lost per human cell per day (reviewed in *3,18,39,40,68*). Because unrepaired AP sites are potentially both cytotoxic and mutagenic (noncoding), this burden of damage to DNA represents one of the major threats to viability and genome stability in human cells. AP sites in DNA can also arise either by the actions of reactive oxygen species (ROS), or by enzymatic excision of damaged bases via the cleavage of the N-glycosyl bond catalyzed by a DNA glycosylase. AP sites in double-stranded DNA are recognized by a class of enzymes termed AP endonucleases that cleave the phosphodiester backbone on the 5′ side of the AP site via a hydrolytic mechanism and hence catalyze the initial step in AP site repair (reviewed in *3,18,68*). A number of DNA glycosylases exhibit AP site-cleavage activity as part of their mechanism of action. However these enzymes act as β-elimination catalysts, cleaving the phosphodiester backbone 3′ to the AP site. This class of enzyme (so-called class I AP endonucleases) will not be discussed further in this chapter. Instead we direct readers to recent reviews containing a discussion of the properties of these enzymes *(13,14,22,37,45,59,69).*

In all organisms that have been analyzed, the hydrolytic AP endonucleases (the class II enzymes) perform roles in addition to AP site repair. In particular, these enzymes act as phosphodiesterases to remove atypical moieties (i.e., other than 3′ hydroxyl) from the 3′ termini of DNA strand breaks induced by ionizing radiation and other DNA damaging agents that generate ROS *(3,16,18,59,68).* These atypical termini, such as 3′ phosphoglycolate and 3′ phosphate, are generated through attack on the sugar moiety of DNA, generating a break in the phosphodiester backbone but leaving a fragment of the sugar at the 3′ terminus of the break. Because DNA polymerases require a 3′-OH terminus to prime DNA repair synthesis, removal of these 3′ blocking lesions is essential to allow repair of oxidative DNA damage to be completed. This chapter focuses on the major AP endonuclease/phosphodiesterase in human cells, called HAP1 (also called APE1, Ref-1, and APEX) (*see 3,6,16,55* for previous reviews). In particular, we shall

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review our current knowledge about the structural basis of AP site recognition and repair by HAP1. A wider review of the base excision repair (BER) pathway in which HAP1 participates is discussed in Chapter 3 by Strauss.

In addition to its ability to recognize and participate in the repair of several classes of DNA lesions, HAP1 also possesses an apparently unrelated activity that can regulate the reduction/oxidation (redox) state of cellular proteins and so modulate their ability to bind to DNA. We shall discuss how this "redox" function might be relevant in the cellular response to oxidative stress.

2. THE HAP1 FAMILY OF AP ENDONUCLEASES

HAP1 belongs to family of structurally and functionally conserved enzymes that has members in bacteria, unicellular eukaryotes, insects, plants, and mammals (Fig. 1). The best-characterized member of the family is *Escherichia coli* exonuclease III (the *xth* gene product; ref *58*), a multifunctional protein that is used extensively in laboratories as a molecular biology reagent. Exonuclease III can be considered to represent the "core" DNA repair-domain characteristic of the family and the only region of the larger eukaryotic family members that is highly conserved. Indeed, this repair domain is one of the most highly conserved protein "modules" yet identified, with at least 50% sequence similarity (when conservative changes are included) evident throughout the family. In addition to HAP1, mammalian-cell homologs of exonuclease III have been identified from bovine and rodent sources *(17,52,53,60,74).* These homologs show a very high level of sequence identity with HAP1 (>90%); the majority of the differences being found within the N-terminal 62 amino acid region.

The largest family member is Rrp-1 from *Drosophila (41,57),* which contains a 427 amino acid N-terminal portion that is not conserved in any other known AP endonuclease (Fig. 1). This domain has been implicated in binding single-stranded DNA and in DNA renaturation. The extensive N-terminal domain of the *Arabidopsis* Arp protein *(2)* is structurally unrelated to that in Rrp-1 and has not been implicated to date in any functions related to DNA metabolism. In contrast, Apn2 and SPBC3D6.10, the recently identified budding and fission yeast homologs of HAP1 *(34),* include a C-terminal domain that is not a feature of exonuclease III or any other known eukaryotic family member (Fig. 1). The role(s) of these C-terminal domains awaits further analysis. The yeast homologs show a lower level of sequence similarity to the other family members, but the key catalytic residues (*see* Subheading 5.2.) are conserved in these enzymes.

HAP1 contains two other functionally defined domains, located N-terminal to the highly conserved DNA repair domain (Fig. 1). The "redox" activity of HAP1, through which it modulates transcription factor function (*see* Subheading 5.), requires residues within the region between amino acids 36 and 82. This domain overlaps the DNA repair domain, which commences at around residue 62. At position 65 within this redox domain is a cysteine residue that has been shown to be essential for redox activity *(67).* A cysteine residue located in an approximately equivalent position is found in the Rrp-1 and Arp proteins, but is absent from the other family members.

The short, extreme N-terminal portion of HAP1 (residues 1–36) is found only in the family members isolated from mammalian species. This domain is essential for the targeting of HAP1 to the nucleus (*see* Subheading 7.).

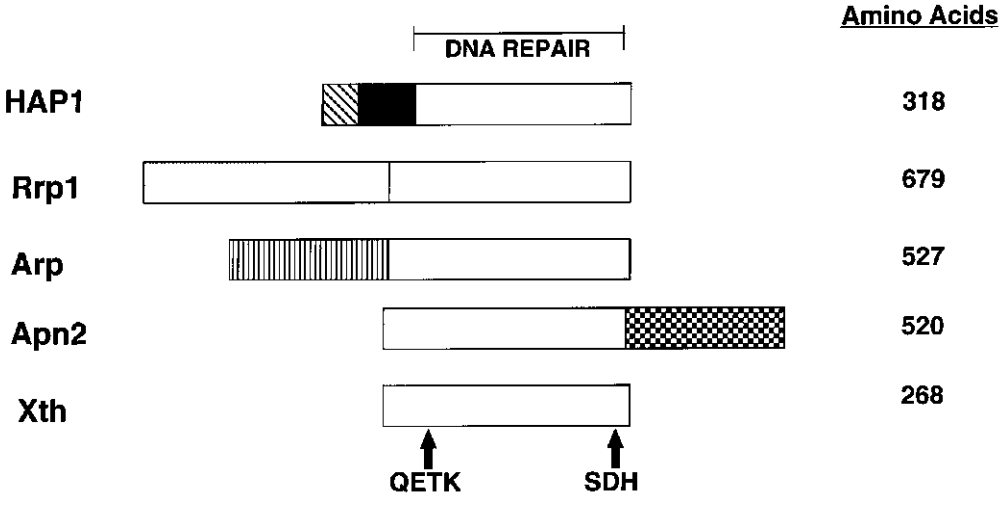

Fig. 1. Schematic representation of the structure of selected members of the HAP1 family of AP endonucleases. The proteins shown are HAP1 *(H. sapiens),* Rrp-1 *(D.melanogaster),* Arp *(A. thaliana),* Apn2 (*S. cerevisiae)* and Xth *(E. coli).* The proteins are aligned by their DNA repair domain, which is shown as an open box and indicated above. The defined "redox" domain of HAP1 is shown as a black box, and the nuclear targeting domain of HAP1 is shown by the diagonally hatched box. The N-terminal domains of Rrp-1 and Arp, which are absent from the other members and are not homologous to each other, are shown with gray shading and vertical hatching, respectively. The C-terminal domain of Apn2, which is absent from the other members is shown as a checked box. The number of amino acid residues in each protein is shown on the right. The positions of the QETK and SDH motifs, which contain the invariant glutamate and histidine residues (Glu-96 and His-309 in HAP1) that are crucial for metal ion binding and catalysis, respectively, are indicated below the Xth protein. See text for details.

3. DNA REPAIR FUNCTIONS OF HAP1

3.1. Substrates for HAP1

Exonuclease III has long been known to be a multifunctional enzyme that possesses AP endonuclease, 3′ phosphodiesterase, 3′ phosphatase, RNaseH, and 3′–5′ exonuclease activities *(24,50,51,54,70).* However, a functional significance for some of these activities remains elusive. In particular, no role for the RNaseH or exonuclease activity has been identified. Exonuclease III is the major AP endonuclease in *E. coli,* and cell extracts from *xth* mutants have much-reduced levels of AP-site nicking activity *(24,70).* Consistent with this, *xth* mutants are sensitive to monofunctional alkylating agents that generate AP sites following glycosylase-mediated excision of alkylated bases *(44).* Evidence that the 3′ phosphodiesterase and phosphatase activities of exonuclease III are important in vivo comes from the finding that *xth* mutants are also sensitive to oxidant chemicals *(15),* particularly hydrogen peroxide, that generate DNA strand breaks terminating in a fragmented sugar group.

In contrast to exonuclease III, for which rates of phosphodiester bond cleavage for its various enzymatic activities are broadly similar, HAP1 can be considered to have evolved as a more specialized repair enzyme. HAP1 is a particularly powerful AP endonuclease, but is 100- to 1,000-fold less efficient (based on catalytic rates) than

exonuclease III as a phosphodiesterase, phosphatase, RNaseH, or exonuclease enzyme *(4,5,10,60,73).* This calls into question the biological significance of these additional activities in HAP1. Nevertheless, our original purification of BAP1, the bovine homolog of HAP1, was on the basis of monitoring 3′ diesterase activity against phosphoglycolate residues generated by bleomycin, and it was clear during those experiments that BAP1 represents the major 3′ phosphoglycolate diesterase activity in extracts from bovine calf thymus *(53).* Similarly, the mouse homolog of HAP1, termed APEX, was purified on the basis of this activity, and APEX has been demonstrated to be the most powerful 3′ diesterase activity in mouse nuclei *(60–62).* Although the 3′ phosphodiesterase activity of HAP1 is likely to have some functional significance, the enzyme is unable to remove 3′ phosphoglycolate residues from a 1 or 2 bp protruding 3′ terminus at a DNA strand break *(64),* suggesting that additional proteins are required for the repair of at least some forms of 3′ blocking lesions. HAP1 is competent, however, for removal of these lesions from blunt-ended or 3′ recessed ends. Consistent with an apparent requirement in human cells for additional 3′ phosphodiesterases, at least one activity distinct from HAP1 that can remove 3′ blocking lesions from oxidatively damaged DNA has been partially purified *(10,75).* However, this enzyme has eluded identification to date.

3.2. HAP1 is a Functional Homolog of Exonuclease III

Expression of the HAP1 cDNA in *xth* (exonuclease III-deficient) and *xth nfo* (exonuclease III and endonuclease IV deficient) strains of *E. coli* has been shown to confer at least partial resistance to DNA-damaging agents, indicative of functional complementation *(11,16,52).* Consistent with a key role as an AP endonuclease rather than as a 3′ phosphodiesterase, HAP1 expression can complement the sensitivity of these mutants to alkylating agents, but not to hydrogen peroxide. Nevertheless, some degree of acquired resistance to γ-rays has been observed in these experiments *(12),* suggesting that HAP1 can function as a phosphodiesterase in vivo.

3.3. Kinetics of AP Site Cleavage by HAP1

The kinetic parameters for cleavage at AP sites by HAP1 have been quantified in several different laboratories. For example Strauss et al. *(63)* studied cleavage at an AP site generated by excision of a uracil residue in an oligonucleotide substrate, and found values for K_m and K_{cat} of 100 nM and 600 min^{-1}, respectively. With our preparations of HAP1 and a similarly generated AP site-containing oligonucleotide substrate, we obtained values of 47 nM for K_m and 150 min^{-1} for K_{cat} *(83).* Erzberger et al. *(20)* have also detailed the kinetics of cleavage at AP site analogs, including the frequently studied tetrahydrofuran (THF) residue, and obtained values for this residue of 27 nM and 40 min^{-1}. In that study, other analogs, such as propanediol and a branched abasic structure, were less efficiently cleaved, with lower K_{cat} values. Thus, there is general agreement that the specificity constant of HAP1 (defined as K_{cat} divided by K_m) is in the range 3–6 min^{-1} nM^{-1} for "regular" AP sites, but is several-fold lower for AP site analogs. These in vitro experiments using oligonucleotide substrates have also shown that in order to recognize AP sites efficiently, HAP1 requires at least 4 bp 5′ and 3 bp 3′ to the lesion. As part of the recognition process, HAP1 makes contacts with both the major and minor grooves and with both the AP site containing strand and the opposing strand of the DNA *(72,73)* (*see* model in Subheading 5.).

3.4. Binding of HAP1 to AP Sites

Treatment of HAP1 with EDTA eliminates AP endonuclease activity, but does not prevent binding of the enzyme to its substrate. This observation has been exploited to study determinants of AP site recognition by HAP1 and the half-life of HAP1:DNA complexes upon addition of Mg^{2+}. Rothwell and Hickson *(56)* and Wilson et al. *(72)* demonstrated that addition of ethylene diamine tetraacetic acid (EDTA) extended the half-life of HAP1:AP site complexes to a degree that permitted detection of the complex using gel-retardation assays. Using this methodology, the highly conserved Asn-212 residue was shown to be important for AP site recognition by HAP1 *(20).* Wilson et al. *(72)* further showed that the phosphodiester backbone in the HAP1:DNA complex is intact, but can be cleaved rapidly upon addition of Mg^{2+}. Consistent with this, Strauss et al. *(63)* calculated the rate of product release to be extremely rapid and proposed that the reaction mechanism for HAP1 follows the scheme proposed by Briggs-Haldane, in which the forward catalytic reaction competes with the dissociation of the enzyme-DNA complex. Recent data have extended this model and suggest that Mg^{2+} plays roles both in catalysis and in substrate interactions *(42).*

3.5. HAP1 Repairs Oxidized AP Sites and Some Bulky DNA Adducts

HAP1 is efficient at repairing not only regular AP sites, but also oxidized AP sites generated by ionizing radiation and radiomimetic chemicals. DNA containing one form of oxidized AP site, the C-4-keto-C-1-aldehyde generated by bleomycin, is cleaved by HAP1 with an efficiency close to that for non oxidized AP sites *(80).*

Several agents, including ionizing radiation, can generate multiple DNA lesions in close proximity. Hence, cleavage at oxidized AP sites generated in this way has the potential to generate double-strand breaks in DNA, a much more cytotoxic lesion than any individual oxidative lesion. Chaudhry and Weinfeld *(9)* showed that the ability of HAP1 to cleave at AP sites closely spaced on opposite strands of a DNA duplex is influenced by the relative positions of the two lesions. AP sites positioned 3′ to each other can be cleaved, albeit less efficiently that single lesions, but those placed 5′ to each other are either not cleaved at all, or only one of the two strands is cleaved efficiently.

Although the BER pathway of which HAP1 is a constituent is generally considered to be specific for "small" lesions in DNA, unlike the nucleotide excision repair pathway, Hang et al. *(27)* showed that HAP1 is involved in the repair of a bulky carcinogenic adduct in DNA. Reaction of *p*-benzoquinone (a benzene metabolite) with DNA generates several lesions including 3,N^4 benzetheno-2′-deoxycytidine (*p*-BQ-dC). This lesion has no associated AP site. HAP1 cleaves DNA 5′ to this lesion, leaving the adduct bound to the 5′ residue of the cleaved DNA. Further work on mutant HAP1 proteins lacking residues important for AP endonuclease activity, indicated that the pBQ-dC endonuclease activity utilizes the same active site as that used for AP site cleavage *(26).*

4. REDOX FUNCTIONS OF HAP1

4.1. The Phenomenon of Redox Regulation of Proteins

Fos-Jun dimerization forms a bipartite DNA-binding domain that interacts with pseudopalindromic AP-1 binding sites, and with palindromic CRE sites *(31).* Regulation of Fos-Jun DNA binding activity has been associated with a "redox" process medi-

ated by the reduction of a conserved cysteine residue located in the DNA binding domain of Fos-Jun heterodimers and Jun-Jun homodimers *(1)*. DNA binding and transcriptional activation by such complexes are inhibited in an oxidizing environment, with functional activity of Fos-Jun heterodimers and Jun-Jun homodimers being restored by the addition of reducing agents such as dithiothreitol (DTT). Substitution of Cys-154 in Fos or Cys-272 in Jun with serine, results in an increase in DNA binding activity (and a concomitant loss of redox regulatory control), suggesting that Fos-Jun activity is mediated by the reduction of cysteine residues *(31)*. A similar redox regulatory system has been shown for p53 (*see* Subheading 4.3.).

4.2. The N-terminal Region of HAP1 Mediates Redox Regulation of Fos-Jun

The connection between the regulatory phenomenon described earlier and HAP1 came from work in the Curran laboratory showing that a factor present in human cells could substitute for DTT in stimulating DNA binding of oxidized Fos-Jun *(76)*. Isolation of cDNAs encoding this factor, termed Ref-1 by these authors, demonstrated that it was identical to HAP1 *(78)*. The region of the HAP1 protein that confers redox activation of Fos and Jun proteins has been mapped in two studies through an analysis of truncated versions of the HAP1 protein *(67,77)*. These studies gave similar results in that C-terminal truncation of HAP1 eliminates DNA repair, but not redox activity, whereas truncation from the N-terminus has the opposite effect. Fine mapping of the region responsible for redox function indicated that the first 36 residues of HAP1 are dispensible, but that the "domain" between amino acids 36 and 82 is essential *(67,77)*. Indeed, our unpublished data indicate that a polypeptide comprising residues 1–82 possesses redox activity, suggesting that residues present in the core DNA repair domain beyond residue 82 are unlikely to be critical for the redox function of the HAP1 protein in vivo.

To date, only one amino acid residue has been implicated in the redox function of HAP1. A site-directed mutagenesis study focusing on the putative role of cysteine residues in HAP1 identified Cys-65 as important for regulation of Jun DNA binding *(67)*. Substitution of Cys-65 by alanine results in a significant loss of HAP1 redox activity. Whether this effect is a direct one, indicating that cysteine-65 might be the redox active site, is unclear at this stage, particularly considering the fact that the crystal structure of HAP1 indicates that cysteine-65 is not surface exposed (*see* Subheading 5.). These data suggest either that a conformational change in HAP1 is required in order for it to direct interactions with transcription factors, or that the negative effect on redox activity of the Cys-65 to alanine substitution is mediated through altering the tertiary structure of HAP1. Further analyses will be required to distinguish between these possibilities.

4.3. p53 is Regulated by HAP1

A great deal of interest has been generated by the finding that p53 is one of the apparent targets for regulation by HAP1. Jayaraman et al. *(33)* purified a protein that could convert a "latent" form of p53 that is inert for DNA binding into a form that could activate transcription in vitro. Analysis of this protein indicated that it is identical to HAP1. Interestingly, these authors suggested that HAP1 plays two roles in the regulation of p53 function, one redox activity-dependent and one independent of this activity. They found that HAP1 can stimulate the DNA binding activity of oxidized forms of

both full-length p53 and a C-terminally truncated version (p53Δ30). However, in the presence of DDT, HAP1 potently stimulates binding by full-length p53, but not by truncated p53. Interestingly, unlike the activation of Fos-Jun dimers, which seems to require stoichiometric amounts of HAP1, stimulation of p53 by HAP1 is surprisingly potent, with half maximal stimulation being observed at a molar ratio of HAP1 to p53 of approx 1:200 *(33).* The functional relevance of this interaction has also been demonstrated by the observation that HAP1 can enhance the ability of p53 to transactivate gene expression *(33).*

4.4. Relevance of Redox Control In Vivo

To date, there have been only a limited number of studies that have addressed the question of whether this form of redox regulation of transcription factors actually exists in vivo and, moreover, whether it is relevant to the regulation of gene expression. One study suggesting that redox regulation is biologically important utilized a mutated Fos protein in which the critical cysteine residue in the DNA binding domain was replaced by a serine *(47).* Retroviral-mediated expression of the serine-substituted Fos protein in chicken-embryo fibroblasts caused a three-fold increase in AP-1 DNA binding activity compared to controls expressing the nonmutated Fos protein. Moreover, viruses expressing the mutant Fos protein gave increased numbers of transformed colonies and a general increase in colony size compared to controls, suggesting strongly that the ability to escape redox control enhances the transforming capabilities of the Fos protein *(47).*

Thioredoxin is a multifunctional protein that can mediate redox control of numerous cellular processes via a cysteine-dependent redox active center. It has been shown that the ability of HAP1 to modulate Fos-Jun DNA binding is enhanced by thioredoxin *(30),* suggesting that thioredoxin may activate HAP1 through its ability to maintain HAP1 in a reduced and consequently active state. Using in vitro cross-linking and a mammalian cell two-hybrid system, Hirota et al. *(30)* demonstrated that HAP1 and thioredoxin are able to associate in a complex and that this association requires the redox active center of thioredoxin. These data suggest that HAP1 and thioredoxin form part of a redox-mediated cascade that is important for regulation of gene expression in mammalian cells.

5. STRUCTURE OF HAP1 AND CHARACTERIZATION OF THE DNA REPAIR ACTIVE SITE

5.1. Structure of HAP1

The crystal structures of HAP1 *(23)* and exonuclease III *(46)* have been determined at 2.2Å and 1.7Å resolution, respectively. Not suprisingly, given their primary sequence similarity, HAP1 and exonuclease III show strong conservation of tertiary structure. In order to generate usable crystals of HAP1 *(23),* it was necessary to delete the N-terminal 35 amino acids. Hence, the discussion of the HAP1 structure given below relates to a molecule comprising residues 36–318 (designated $HAP1^{36–318}$). This truncated version of HAP1 is, however, fully functional, with no apparent reduction in AP endonuclease or redox activity *(67).* $HAP1^{36–318}$ in the presence of the calcium analog, samarium, produced high-quality crystals *(23).*

With the exception of the region between residues 36 and 43, $HAP1^{36–318}$ is a globular α/β protein consisting of two domains (domain 1. residues 44–136 and 295–318.

domain 2. 137–260 and 282–294), with overall dimensions 40 Å × 45 Å × 40 Å. Both domains display similar topologies, each comprising a six-stranded β-sheet surrounded by α-helices, which pack together to form a four-layered α/β-sandwich. Each β-sheet is composed of a pair of three-stranded anti-parallel β-motifs, with the β-strands in both domains being flanked by topologically equivalent α-helices. Strands β_1/β_7 are flanked by kinked α-helices, α_1–α_2/α_5–α_6, in domains 1 and 2, while β_3/β_8 are flanked by short α-helices, α_3/α_4, respectively. Helices α_7, – α_{10} have no comparable structure in domain 1, while α_{12} forms an inter-domain helix. Residues 261–281 form two extra-domain anti-parallel β-strands, β_{10} and β_{11}, separated by a helical turn, α_{11}.

An important insight into the catalytic mode of action of HAP1 came from the finding that its structure is also very similar to that of bovine DNase I *(23).* This is despite the fact that DNase I and HAP1 show <20% primary sequence homology. HAP1, exonuclease III and DNase I not only illustrate an overall conservation of the protein fold (Fig. 2), but also a conservation of residues shown by site-directed mutagenesis to be important for catalysis (*see* Subheading 5.2.). It is clear from these comparisons that the mechanism by which these enzymes cleave the phosphodiester backbone of DNA is probably very similar. We have proposed a model *(23)* for how HAP1 binds to DNA based on the structure of DNase I bound to a DNA octamer duplex *(38).* In this model, HAP1 is proposed to make only a limited number of specific protein/base interactions, and instead to bind DNA mainly via phosphate and nonspecific hydrophobic interactions.

5.2. Active Site of HAP1 and Proposed Catalytic Mechanism

The active site of HAP1 lies in a pocket at the base of the α/β-sandwich and is surrounded by loop regions. Within the active site, the imidazole ring of His-309 interacts with the carboxylate of Asp-283, which in turn forms a hydrogen bond with Thr-265. The side chains of Tyr-171 and Glu-96 are hydrogen bonded, as are those of Asn-68 and Asp-210, with the side chain of Asp-210 also being hydrogen-bonded to the main-chain amide of Asn-212. Together with the hydrogen bonding of Asp-70 to Lys-98, these interactions form an intimate hydrogen-bonding network within the active site. A single metal ion binds to the side-chain of Glu-96 (mean distance of 2.5Å from the carboxyl group).

During catalysis, it is proposed that His-309 of HAP1 acts as the general base to abstract a proton from a water molecule, whereas Asp-283 orientates the imidazole ring of His-309 and stabilizes its transiently positive-charged state (Fig. 3). The resulting hydroxide ion then attacks the scissile AP 5′-phosphate via an inversion of configuration. The catalytic importance of His-309 and Asp-283 have been demonstrated, because mutation of His-309 to Asn and Asp-283 to Ala results in a dramatic reduction in catalytic activity *(4).* However, recent mutagenesis work has suggested that Asp-283 is not essential for AP endonuclease activity and that its role may be substituted by Asp-308, because the double mutant is markedly more defective in AP endonuclease activity than is either single mutant *(43).* This is possible, because the carboxyl group of Asp-308 is only 3.2Å from His-309. The transition state intermediate could be stabilized by the divalent metal ion bound to Glu-96, in a similar way to that proposed for DNase 1 *(71).*

Recently, Mol *et al. (82)* reported the cocrystal structure of HAP1 bound to oligonucleotides containing a single, synthetic abasic site (tetrahydrofuran). Their data provide new insights into both the catalytic mechanism of action and the mode of AP site recognition by HAP1 (*also see* discussion below). Mol *et al.* propose that the hydroxyl nucle-

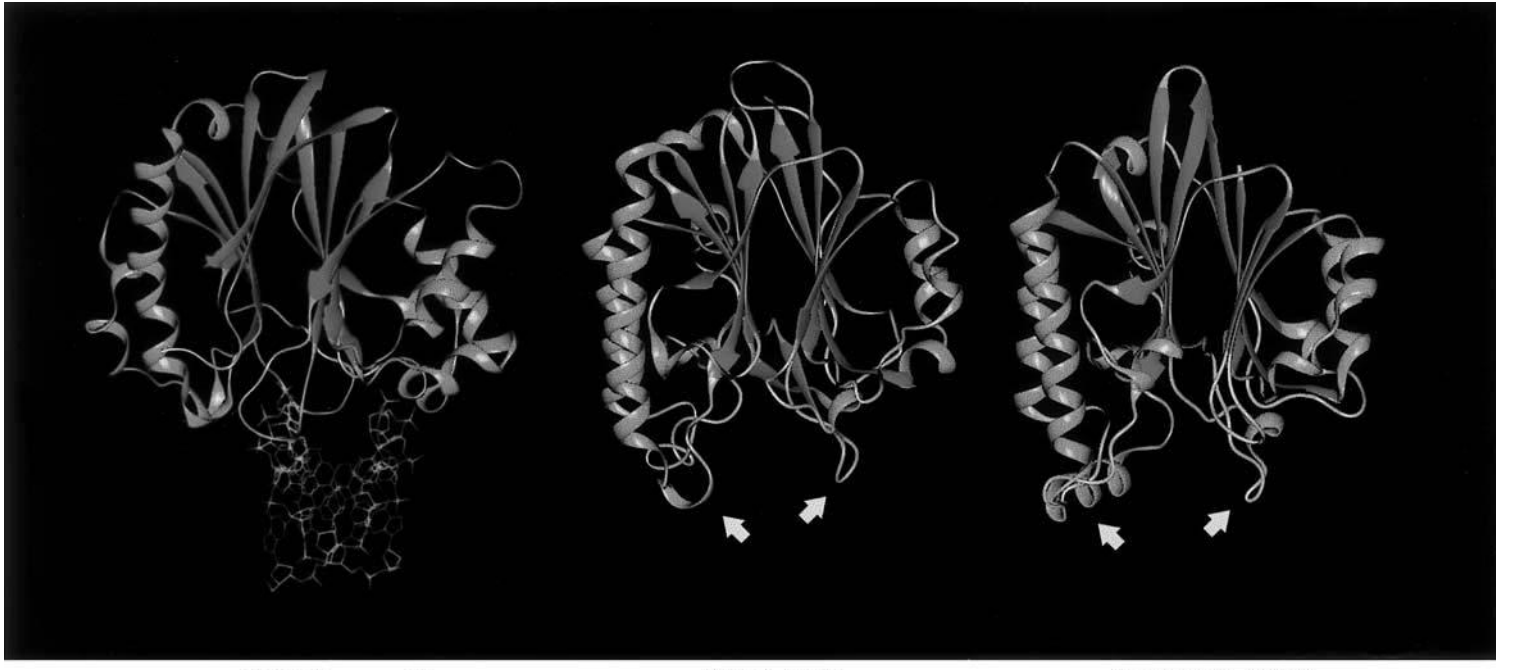

Fig. 2. Structural similarity between the overall folds of DNase I, HAP1, and exonuclease III. Ribbon representation of DNase I, HAP1, and exonuclease III, with α-helices colored blue and β-strands colored pink. DNase I is shown complexed to DNA, (atom colored stick model). The yellow arrows indicate the three helical loop regions that are present in HAP1 and exonuclease III, but are absent from DNase I (*see* text). Figure produced using PREPI (courtesy of S. Islam and M Sternberg, ICRF).

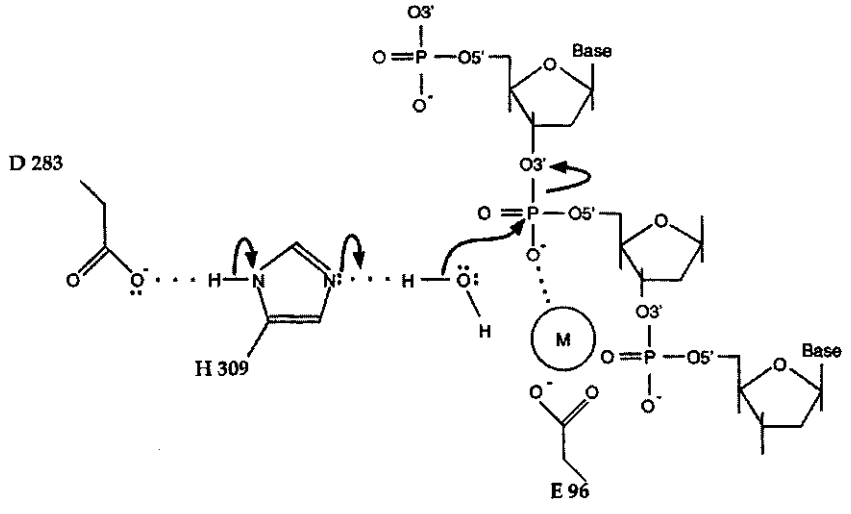

Fig. 3. Proposed catalytic mechanism for HAP1. His-309 acts as general base and abstracts a proton from a water molecule. The hydroxide ion thus formed then attacks the phosphate group 5′ to the AP site. A divalent metal ion (M), bound to Glu-96, could be involved in stabilizing the transition state intermediate.

ophile is generated by the Asp-210 residue, which is oriented by hydrogen bonding with Asn-212, and Asn-68, and that His-309, Asn-212 and Asn-174 act to correctly orient the target AP site 5′ phosphate. In this scheme, the role of the metal iron is to stabilize the transition state intermediate and the O3′ leaving group. Consistent with this proposed scheme, mutation of Asp-210 to Ala or Asn eliminates AP endonuclease activity *(83,84).* Nevertheless, these mutated proteins are still competent for AP site binding *(83,84).*

5.3. AP Site Recognition by HAP1

The solution structures of several oligonucleotides containing various AP site analogs have been determined by nuclear magnetic resonance (NMR). By superimposing the scissile phosphodiester bond located at the active site of HAP1 from the model by Gorman et al. *(23)* on the averaged NMR structure of DNA containing an αAD (apyrimidinic duplex) moeity *(7),* potential interactions between HAP1 and the DNA duplex can be proposed. In this model (Fig. 4), the helical loops α5 (residues 176–181) and α11 (residues 267–277) are positioned within the major and minor grooves, respectively, making potential DNA-phosphate backbone interactions. A third region, α8 (residues 222–227; 164–173 in exonuclease III) is also positioned within the major groove. Hydrophobic residues conserved in the AP endonuclease family (Leu-220, Phe-232, and Trp-280 in HAP1), seem to be important for stabilizing and/or positioning these loops. Interestingly, these three loops are not conserved in DNase I and have been proposed *(23)* to be a major determinant of the AP site specificity of HAP1 and exonuclease III, given that DNase I is a nonspecific endonuclease.

The primary sequence of HAP1 also shows similarity to L1 endonuclease (*see* discussion in ref. *23*), which forms part of human L1 elements, a highly abundant poly(A) (i.e., non-LTR) retrotransposon that contains highly repetitive DNA. L1 endonuclease,

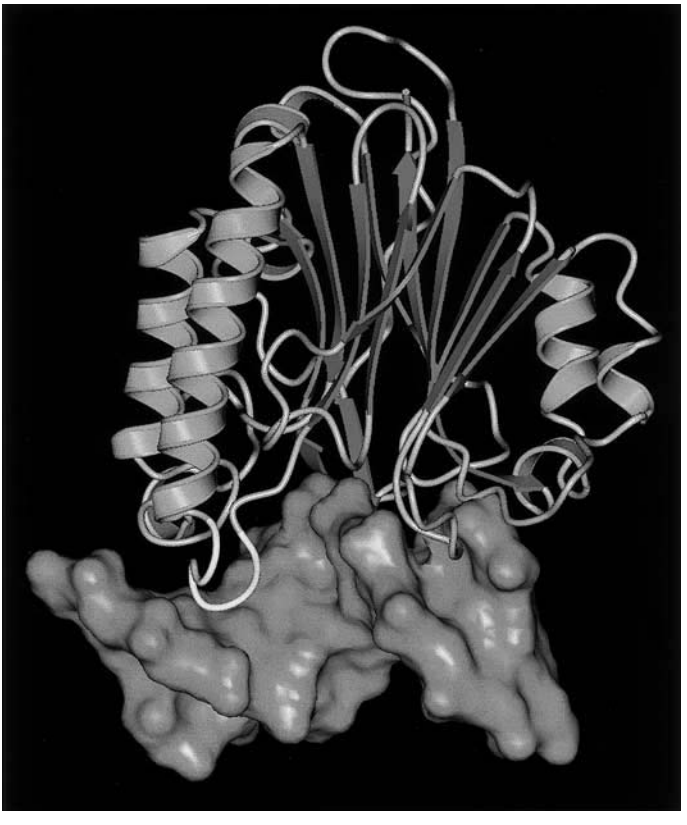

Fig 4. Model of HAP1 bound to DNA. Ribbon representation of HAP1 with α helices shown in blue and β sheets in pink. Apyrimidinic duplex DNA (shown in the a hemiacetal conformation and taken from Berger and Bolton *[7]*) is represented as a gold surface generated using a probe size of 1.4 Å. Loop regions are predicted to lie in both the major and minor grooves of the DNA. The loop region α8, homologous to the one observed by Cal *et al. (8)* to impart AP endonuclease-like activity on Dnase I, is colored yellow. Figure produced using PREPI (courtesy of S. Islam and M Sternberg, ICRF).

like DNase I, has no specificity for AP sites, and 2 of the 3 helical loops specific for the AP endonucleases (α8 and α11) are absent from L1 endonuclease. To address the functional relevance of the α8 loop, Cal et al. *(8)* inserted the equivalent loop from exonuclease III (designated α_M) into DNase I. The modified DNase I (DNase-exohelix) acquired the ability to bind and cleave DNA at AP sites *(8),* albeit with a much lower efficiency than that seen with "native" AP endonucleases.

In HAP1, Phe-266 lies close to the active site and has been suggested to be important in the direct interaction with the ribose ring at AP sites *(23).* However, a Phe-266 –Ala mutant shows only a six-fold reduction in AP site-binding activity, suggesting that it is important, but not essential for AP site recognition *(20).* Given that AP sites containing nonring structures are recognized and cleaved by HAP1 *(73),* together with the results of Erzberger et al. *(20),* it seems likely that a number of other structural elements (in addition to the Phe-266 residue) found in the AP endonucleases, but not in DNase I, will be required for efficient recognition and repair of AP sites.

The cocrystal structure of HAP1 with tetrahydrofuran residues *(82)* has provided a novel and direct insight into the mode of AP site recognition by this enzyme. HAP1 inserts loops into both the major and minor grooves of DNA and, as predicted previously *(23),* binds flipped-out abasic deoxyribose in the active site. This stabilization of the flipped moiety is achieved by kinking of the DNA helix. Interestingly, the active site pocket created excludes one of the two racemic forms of the AP site, the β-anomer. The enzyme is locked onto the damaged DNA by insertion of Arg-177 into the major groove and Met-270 into the minor groove. Apparently at odds with these observations is the finding that substitution of Arg-177 or Met-270 by Ala does not eliminate endonuclease activity. Indeed, the R177A mutant has a 3-fold increased K_{cat}. Mol *et al*. *(82)* argue that this apparent anomaly reflects the greater importance attached to coordinating AP site cleavage with the subsequent steps of base excision repair, than to maximizing the rate of enzyme turnover. It may be important for HAP1 to remain bound to the product of its hydrolysis reaction, and not to dissociate from the nicked (and probably toxic) DNA intermediate.

5.4. Structure of the Redox Domain of HAP1

The "redox" domain of HAP1 is associated with the N-terminal region, including amino acids 43–62 *(67,77).* The structural analysis of HAP1 *(23)* shows that this region forms an extended loop which lies across the β-strands, β13 – β14. Cys-65 is also implicated in the "redox" activity of HAP1 *(67).* In the HAP1 crystal structure, Cys-65 is located on β1, with its side-chain pointing into a hydrophobic pocket (containing Trp-67, Trp-75, Trp-83, Pro-89, Leu-92, Leu-94, and Pro-311) and away from the central β-sheet. Solvent accessibility calculations with a probe size of 1.4 Å showed that Cys-65 is inaccessible to solvent and would, therefore, be unlikely to interact directly with residues from other proteins. Interestingly, Cys-93 and Cys-208 lie within the core β-sheet, with their side-chains only 3.5 Å apart, and are in close proximity to Cys-65. However, in the crystal structure, there is no evidence for the existence of a disulfide bond between them. However, incubation of diamide with HAP1 has been associated with the formation of an intramolecular disulfide bond *(77),* suggesting that Cys-93 and Cys-208 could form a disulfide bond under different crystallization conditions.

Removal of 50 or more N-terminal residues from HAP1 abolishes redox activity *(67,77).* Such truncations could be deleterious to the overall HAP1 fold and may account, at least partially, for the lack of redox activity. Several residues, particularly in the region comprising residues 47–59, make hydrogen bonding or salt-bridge interactions with the globular core of the enzyme *(23).* The role of Cys-65 in mediating the "redox" activity is perplexing, because it is buried in a hydrophobic pocket. However Cys-65 substitutions could disrupt this large hydrophobic-core region, adversely affect the stability and/or folding of HAP1, which then indirectly influences the apparent "redox" activity by altering the conformation of the N-terminal domain. It is also possible that the HAP1 crystal structure reported by Gorman et al. *(23)* is not representative of a "redox" active conformation. HAP1 must be in a reduced state to exhibit redox activity, and no attempt was made to maintain such a state during crystal growth. A reducing environment may promote a conformational change, which could then allow Cys-65 to become solvent-exposed.

6. ROLE OF HAP1 IN CELLULAR PROTECTION AGAINST CYTOTOXIC STRESSES

Targeted disruption of the gene encoding the murine HAP1 homolog results in early embryonic lethality *(79)* and therefore it has not been possible to derive any information from a mouse "knockout" model for HAP1 deficiency. As an alternative, several groups have studied cell lines in which HAP1 has been depleted following expression of *HAP1* antisense RNA. These studies have shown that HAP1 is required for cellular protection against a wide range of DNA damaging agents and oxidative stresses *(12,48,66)*. HAP1-depleted cells are sensitive to agents that generate AP sites via DNA methylation (such as methyl methane sulphonate; MMS), and to agents that generate oxidative DNA damage, such as x-rays, hydrogen peroxide, paraquat, bleomycin, and hyperoxia (95% O_2). These data are consistent with the known roles of HAP1 as an AP endonuclease and a 3′ phosphodiesterase (*see* Subheading 3.). Perhaps more surprisingly, HAP1-depleted cells are hypersensitive to hypoxic stress (1% O_2). However, the precise mechanism by which hypoxia kills cultured mammalian cells has yet to be delineated, and therefore it is not clear whether the mode of killing HAP1-depleted cells by hypoxia is in any way unique.

Unfortunately, all of the previous studies in this area have failed to discriminate between a DNA repair role and redox role for HAP1 in protecting cells against cytotoxic stresses. Indeed, the vast majority of the agents to which HAP1-depleted cells are sensitive are oxidizing agents that have the potential both to generate oxidative DNA damage and to modulate the redox status of cellular proteins. The finding that HAP1-depleted cells are hypersensitive to MMS, which, as far as we are aware, does not generate any oxidative stress, suggests that DNA repair deficiency underlies at least this phenotypic effect of disrupting HAP1 function. Nevertheless, it would not be surprising if a combination of deficiency in DNA repair and redox functions were important for the pleiotropic radiation and drug sensitivity of cells expressing HAP1 antisense RNA. Evidence to substantiate this suggestion will only be forthcoming when targeted mutations in *HAP1* or one of its mammalian homologs are generated that inactivate separately either the redox function or the repair function.

7. SUBCELLULAR LOCALIZATION OF HAP1

7.1. Immunohistochemical Staining for HAP1

Although the putative redox function for HAP1 could theoretically be performed in any cellular compartment, it is clear that HAP1 must accumulate in the nucleus to effect its role as a DNA repair enzyme. Despite this, a number of immunohistochemical studies have shown that HAP1 is not necessarily exclusively nuclear, but instead is sometimes distributed in both the nucleus and cytoplasm, or indeed can be exclusively cytoplasmic in some cell types. For example, Kakolyris et al. *(36)* studied HAP1 expression level and subcellular localization in normal colorectal mucosa, as well as in hyperplastic polyps, adenomas, and carcinomas of the colon. This study indicated that HAP1 is located in different cellular compartments depending on the extent of differentiation of the cells. In the poorly differentiated cells located in the lower portion of colonic crypts, staining for HAP1 is predominantly nuclear, whereas it is cytoplasmic in the more differentiated superficial colonic epithelium. Moreover, HAP1 is partially

or exclusively localized to the cytoplasm in 90% of adenomas and carcinomas of the colon, and in stromal macrophages. HAP1 staining is exclusively nuclear in stromal fibroblasts and endothelial cells. A similar pattern of marked changes in subcellular localization depending on differentiation state of the cells has been reported by Duguid et al. *(19)* in a study of duodenal tissue. These authors showed that HAP1 is localized to the nuclei of cells in the duodenal crypts and proximal villi, but not in the distal villi. Thus, differential localization of HAP1 is seen in cells from both the proximal and distal intestine.

In a separate study, Kakolyris et al. *(35)* showed that HAP1 has a uniformly nuclear localization in normal breast epithelium, but it is predominately cytoplasmic in lactating and glandular epithelium. In breast carcinomas, HAP1 expression in the nucleus seems to be related to advanced differentiation, which is well-established to relate to good prognostic features. Consistent with those findings, evidence of exclusive localization of HAP1 to the nucleus significantly correlates with negative lymph-node status. In contrast, cases with cytoplasmic as well as nuclear staining showed an association with lymph-node positivity and consequently a poorer prognosis *(35).*

7.2. The Nuclear Localization Signal Sequence of HAP1

Despite the earlier discussion, in our hands transfection of the HAP1 cDNA into cultured human tumor-cell lines results in a predominantly nuclear localization of the protein. This has permitted us to define the amino acid residues in HAP1 that are required for targeting of the protein to the nucleus (unpublished results). We and others have noted the sequence similarity between the extreme N-terminal region of HAP1 and nuclear localization signal (NLS) sequences found in a variety of nuclear proteins. In HAP1, two basic clusters of residues, PKRGKK (residues 2–7) and KKSK (residues 24–27) fit the consensus for a bipartite NLS sequence. In order to confirm that these residues are important for nuclear targeting, we analyzed the effect of mutating or deleting residues within these motifs. Effects on nuclear targeting were detected by transfection of the modified cDNAs into HeLa cells followed by immunofluorescent detection of HAP1. Deletion of residues 1–36 prevents nuclear localization, confirming that the extreme N-terminal portion of HAP1 is vital for targeting of the protein to the nucleus. Mutation of lysines 24 and 25 in the KKSK motif also prevents nuclear localization, but mutation of all 4 basic residues in the PKRGKK motif does not, initially suggesting to us that the KKSK motif might represent the sole NLS sequence in HAP1. Nevertheless, this proved to be incorrect because a truncated HAP1 protein lacking residues 1–20, but retaining the KKSK motif, is not translocated to the nucleus. At this stage, the amino acids within this 20-residue N-terminal leader region that contribute to nuclear targeting are not defined, but may include the basic residues of the PKRGKK motif together with flanking residues.

8. REGULATION OF HAP1 GENE EXPRESSION

The *HAP1* mRNA is expressed constitutively at a relatively high level in many cell lines and tissues. Nevertheless, recent evidence indicates that certain cytotoxic agents can cause a modest increase in the level of HAP1 protein. This “induction” appears to be accompanied by an increase in cellular AP endonuclease activity. Ramana et al. *(49)* showed that *HAP1* mRNA peaks around 9–15 h after exposure of HeLa cells to

HOC1, and can also be induced by hydrogen peroxide, ionizing radiation, and bleomycin. This apparent response to different oxidants is abrogated by treatment of the cells with N-acetylcysteine, suggesting a role for ROS in the induction process. These authors also addressed whether the induction of HAP1 leads to protection against a subsequent challenge by a genotoxic agent (i.e., ROS an adaptive response). When analyzed 12 h after exposure to HOC1, HeLa cells showed an approx two-fold greater level of resistance to hydrogen peroxide, bleomycin, and MMS, but not to γ-rays or UV light. In a similar study using CHO cells Grösch et al. *(25)* showed that *HAP1* mRNA is induced by treatment with hydrogen peroxide and sodium hypochlorite. This induction of HAP1 is associated with an adaptive response to sodium hypochlorite in that it can confer some degree of resistance to a subsequent challenge by hydrogen peroxide. These authors also showed that transient transfection of the *HAP1* cDNA into CHO cells, which leads to an increase in cellular HAP1 levels, reduces the clastogenic effects of hydrogen peroxide. Consistent with *HAP1* being an oxidant-inducible gene, work by Fung et al. *(21)* showed that crocidolite asbestos induces HAP1 protein. This oxidant is important in the pathogenesis of mesothelioma in humans caused by exposure to asbestos.

Although the previous discussion seems to indicate that HAP1 is an inducible protein that confers an adaptive response to certain genotoxic agents, this effect has not been universally observed. For example, during their analysis of the promoter of the *HAP1* gene, Harrison et al. *(28)* failed to detect *HAP1* mRNA inducibility in HeLa cells by paraquat and bleomycin, among other oxidants. Moreover, Tomicic et al. *(65)* showed that overexpression of HAP1 in CHO cells does not confer resistance to MMS or hydrogen peroxide, apparently in contradiction to other published work from the same laboratory *(25,* cited earlier). Despite this, a recent paper by Herring et al. *(29)* showed that levels of HAP1 are associated with intrinsic radiosensitivity in cervical tumor cells, suggesting that the level of this enzyme may be important for cellular protection against x-rays in vivo.

Two studies have shown that hypoxic stress can stimulate expression of the HAP1 protein. Using HT29 cells Yao et al. *(81)* showed that *HAP1* mRNA is induced rapidly during hypoxic stress via a mechanism involving increased gene transcription. Moreover this leads to an accumulation of HAP1 protein that persists for several hours following return of cells to a normoxic environment. In our study, HAP1 protein was shown to be induced within 4 h of exposure to conditions of 1% oxygen and to peak at around 24 h *(66).*

One study has indicated that the HAP1 protein may regulate its own synthesis. During an analysis of HAP1 promoter activity, Izumi et al. *(32)* identified 2 nCARE-like sequences (negative Ca^{2+} response elements) that are responsible for inhibition of promoter activity. Mobility-shift assays showed that HAP1 protein itself binds to this nCARE sequence, suggesting that HAP1 may contribute to negative regulation of its own synthesis.

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5

Mating-Type Control of DNA Repair and Recombination in *Saccharomyces cerevisiae*

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1. INTRODUCTION

Mating type in the yeast *Saccharomyces cerevisiae* can be one of three types: **a**, α, and **a**α. These mating types reflect information present at *MAT,* which is normally *MAT***a** or *MAT*α in haploid cells, and *MAT***a**/*MAT*α in diploid cells. *MAT*α and *MAT* a each have two open reading frames, but functions have only been identified for three gene products, Mat**a**1p, Matα1p, and Matα2p. The *MAT* gene products are key regulators of the different stages of the yeast life cycle (reviewed in refs. *26,28,30*). The Mat**a**1p/Matα2p complex in diploid cells represses transcription of haploid-specific genes, including the repressor of meiosis, *RME1,* and *HO.* In *MAT*α haploid cells, Matα1p complexes with Mcm1p to activate α-specific genes. Matα2p complexes with Mcm1p (as well as Tup1p and Ssn6p) to repress a-specific genes. *MAT* controls mating: **a** cells mate with α cells but not with **a** cells, and vice versa, and **a**α cells do not mate with any of the three cell types. The mating behavior of **a** and α cells reflects their expression of the haploid-specific mating pheromones, **a**- and α-factor and the cognate transmembrane receptors. *MAT* also controls meiosis and sporulation; *MAT***a**/*MAT*α diploids can carry out meiosis whereas diploids expressing only one of the two alleles do not. The ability of **a**α diploids to enter meiosis depends on repression of the regulatory gene *RME11,* which is turned off by the action of the Mat**a**1p-Matα2p repressor complex. This leads to the expression of many meiosis-specific genes. Although natural **aa** or αα diploids are rare, they can be easily produced by using a regulated source of HO endonuclease *(29)* or by deleting one *MAT* allele (i.e. αΔ or aΔ) *(44).*

a and α information is also present at *HMR***a** and *HML*α, but in wild-type cells, these loci are silenced by the products of four *s*ilent *i*nformation *r*egulator *(SIR)* genes acting on sequences adjacent to *HMR***a** and *HML*α *(27,64).* Mating-type switching occurs in haploid mother cells following expression of HO endonuclease, which introduces a double-strand break (DSB) into *MAT,* thereby stimulating a gene-conversion event that transfers information from *HMR***a** or *HML*α to *MAT* (Fig. 1). Considerable effort has been directed toward developing molecular descriptions of the structure, expression, and silencing of mating-type loci, the functions of *MAT* gene products, the regulation of mating-type switching, and the recombinational mechanism that effects the switch *(26).*

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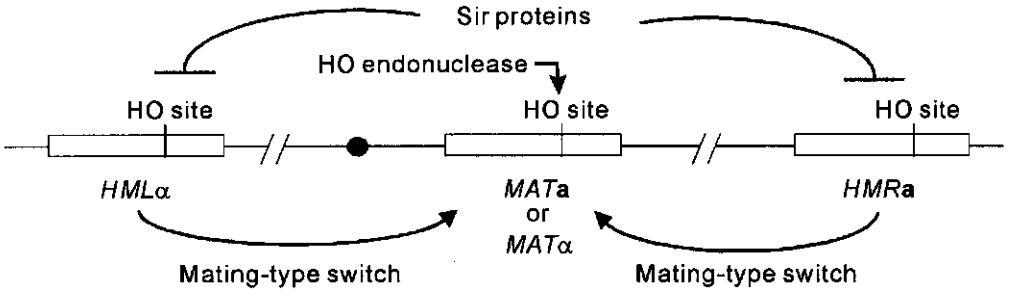

Fig. 1. Mating-type loci in yeast. *MAT* is an expressed locus on chromosome III and encodes either **a** or α information. Within the *MAT* locus is an HO recognition site; cleavage at this site stimulates gene conversion with the largely homologous *HML*α or *HMR***a** loci as donors, converting *MAT*α to *MAT***a** and vice versa. Sir proteins silence *HML*α and *HMR***a** and also block HO endonuclease cleavage at these loci.

This chapter focuses on *MAT* control of various aspects of general DNA repair and recombination, including radiation resistance, spontaneous and damage-induced homologous recombination, and nonhomologous end-joining (NHEJ). Another DNA repair process controlled by *MAT* is donor choice during mating-type switching; this topic was reviewed recently *(25)* and is discussed only briefly here.

2. DNA DOUBLE-STRAND BREAK REPAIR IN YEAST

DNA damage results from spontaneous chemical decomposition (e.g., deamination), chemical reactions of DNA with products of normal cell metabolism (e.g., oxidation), and exposures to exogenous agents including radiation and genotoxic chemicals. There are many types of DNA damage; each type can be classed as single- or double-strand damage. Single-strand damage, such as abasic sites, ultraviolet (UV)-induced pyrimidine dimers, and ionizing radiation (IR)-induced base damage can be repaired by base-excision and/or nucleotide-excision repair pathways, mediated in yeast by genes in the *RAD3* and *RAD6* epistasis groups. Single-strand damage also can be repaired by direct reversal, such as religation of single-strand breaks (SSBs), or photoreversal of pyrimidine dimers by photolyase. Although single-strand damage can stimulate recombination, this stimulation is not necessarily a consequence of the repair process. For example, UV-induced recombination is reduced when UV repair is enhanced, and there is evidence that single-strand damage stimulates recombination only after being converted to DSBs (*see* Chapter 13 and ref. *20*).

In yeast, the repair of double-strand damage (DSBs, interstrand crosslinks) often involves homologous recombination mediated by proteins encoded by genes in the *RAD52* epistasis group (reviewed in ref. *64,* and Chapter 16, Vol. 1). This group includes Rad51p, an *Escherichia coli* RecA homolog with strand exchange/pairing activities; Rad52p, a DNA end-binding protein; and Rad54p, an ATPase with putative helicase activity. Both Rad52p and Rad54p interact with Rad51p and stimulate the strand-exchange activity of Rad51p. Mammalian homologs have been identified for each of these proteins, and the interactions between them also appear to be conserved among eukaryotes (*see* Chapter 15). There are several distinct modes of homologous recombination, including conservative processes such as gene conversion and crossing

over, and the nonconservative process termed single-strand annealing (SSA) that operates between direct repeats. All of these events can result from DSB repair, with the particular outcome(s) dependent on the configuration of the recombining regions. An alternative mechanism of DSB repair is NHEJ, which can be imprecise and hence mutagenic, leading to deletions or insertions, or precise ("direct religation") when DSBs have cohesive, ligatable ends, such as those produced by nucleases. NHEJ is *RAD52*-independent, requiring *YKU70 (HDF1)* and *YKU80 (HDF2),* a special DNA ligase (DNA ligase 4), and its associated Xrcc4 protein (*LIG4* and *LIF1,* respectively, in *S. cerevisiae*) *(13).* yKu70p and yKu80p form the Ku heterodimer that has strong DNA end-binding activity. Yeast lacks the DNA-PKcs protein with which Ku proteins are associated in mammalian cells. Other genes that are important in NHEJ in budding yeast, but apparently not essential in either fission yeast or chicken DT40 cells include *RAD50, XRS2, MRE11 (88,95).* As with homologous recombination proteins, NHEJ proteins are conserved from lower to higher eukaryotes *(13).*

In mammalian cells, NHEJ is an important DSB repair mechanism involved in V(D)J joining and the repair of IR-induced DSBs. Because homologous recombination is so efficient in yeast, it was only recently recognized that NHEJ also has important roles in DNA repair. There are interesting differences between NHEJ in yeast and mammalian cells. For example, imprecise NHEJ is very rare in yeast *(41,55,61,71,72),* but quite common in mammalian cells *(47).* In mammalian cells NHEJ is effective for repairing both IR- and nuclease-induced DSBs since Ku mutants are sensitive to both types of DSBs (*see* Vol. 2, Chapter 17). In contrast, yeast *yku70* mutants have wild-type resistance to IR and nuclease-induced DSBs as long as homologous recombination is functional *(60,76).* Cells sensitive to IR generally show cross-sensitivity to the radiomimetic agents methylmethane sulfonate (MMS) and bleomycin. It is interesting that haploid yeast Rad^+ Ku^- strains are resistant to IR, but show mild sensitivity to MMS *(56,60)* and marked sensitivity to bleomycin *(53).* Apparently, a fraction of MMS and bleomycin damage cannot be repaired by homologous recombination in haploid cells, but instead is repaired by a Ku-dependent (NHEJ?) pathway. In contrast to the haploid MMS results, a diploid Ku^- strain showed no MMS sensitivity *(76),* consistent with enhanced homologous recombination in diploids (*see* Subheading 3.1. and 3.7.). Even in a *rad52* background where homologous recombination is effectively absent, *yku70* mutation confers only slightly more IR sensitivity, indicating that IR-induced DSBs are rarely processed by NHEJ in yeast *(76).* In contrast, nuclease-induced DSBs are efficiently repaired by (precise) NHEJ (*see* Subheading 3.6.). Thus in yeast, homologous recombination and NHEJ compete for repair of nuclease-induced DSBs, but most or all IR-induced DSBs are processed by homologous recombination. This accounts for the marked IR sensitivity of G1 haploids as these lack homologous repair templates (*see* Subheading 3.1.). The different repair efficiencies for nuclease- and IR-induced DSBs by NHEJ lead to interesting differences in the effects of *MAT* on homologous recombination stimulated by these different types of DSBs, as discussed in Subheading 3.

3. *MAT* CONTROL OF RADIATION RESISTANCE AND RECOMBINATION

3.1. Distinction between Effects of Ploidy and MAT Heterozygosity on Radiation Resistance

It was established very early that diploid yeast cells are more resistant to the cytotoxic effects of IR than haploid cells *(43).* Similarly, haploid G2 cells are more radiore-

sistant than G1 cells *(11,68).* These results could reflect the availability of homologous chromosomes or sister chromatids that act as repair templates for homologous recombination. Although this is a reasonable idea, it was recognized early that heterozygosity at *MAT* was responsible for at least part of the increased resistance because **a**/α diploids were more radioresistant to IR than **aa** or αα diploids *(62).* The higher IR resistance of **a**α diploids was confirmed in many subsequent studies *(17,21,31,34,36,51,70,73).* The effect of *MAT* heterozygosity is seen in both G1 and G2 cells, but more marked effects are seen in G1 *(36).* Although radioresistance generally increases with increasing DNA content, tetraploids are actually less radioresistant than diploids *(36,62).* In contrast to the diploid results, *MAT* heterozygosity in haploid cells does not increase radioresistance *(42),* suggesting that the diploid *MAT* effects reflect enhanced homologous recombination that is not possible in haploids (i.e., in G1 cells). Haploid strains are normally homozygous at *MAT* but can be made heterozygous by introducing a second copy of *MAT* on a circular plasmid, or by activating *HML*α and *HMR***a** by disabling a *SIR* gene. Together these results clearly indicate that resistance to IR is influenced independently by ploidy and *MAT* genotype.

Saeki et al. *(70)* connected the radioprotective effect of *MAT* heterozygosity to DSB repair and recombination by showing that mutations in genes involved in recombinational repair *(RAD51, RAD52,* and *RAD54)* abolished the radioprotective effect. Two other important recombination genes are *RAD55* and *RAD57,* which encode proteins of a heterodimer *(35,74,79).* Interestingly, deletions of these genes cause radiosensitivity only at low temperature and this defect can be suppressed by overexpression of *RAD51 (35),* and by *MAT* heterozygosity *(21,51).* In contrast, cells defective in either excision repair (*RAD3* epistasis group) or error-prone repair (*RAD6* epistasis group) still display greater radioresistance when *MAT* is heterozygous *(31,70).* The idea that *MAT* heterozygosity influences radioresistance through effects on homologous recombination was further supported by the finding that the radioresistant phenotype of *rad52-20* mutant cells was suppressed by heterozygosity at *MAT* (as well as by overexpression of *RAD51*). *rad52-20* haploids and *MAT* homozygous diploids show marked sensitivity to IR, but haploid *rad52-20 sir* double mutants, and *MAT* heterozygous diploids display essentially wild-type radioresistance *(73).* Thus, the protective effects of *MAT* heterozygosity to IR damage reflect, at least in part, enhanced homologous recombination (*see* Subheadings 3.5. and 3.7.).

3.2. Effects of MAT on Resistance to UV and MMS

In contrast to the results with IR, several studies showed that *MAT* status had little or no effect on UV resistance in Rad$^+$ cells *(18,21,50).* However, in mutants sensitive to killing by UV, *MAT* heterozygotes were markedly more resistant to killing by UV than *MAT* homozygotes. In fact, some mutants defective in excision or error-prone repair pathways, such as *rad18,* display even stronger *MAT* effects on UV resistance than those seen in Rad$^+$ cells with IR. These results can be explained by the idea that some damage normally processed by the UV repair pathways can be channeled into the recombinational repair pathway; see Heude and Fabre *(31)* and references therein. It was also shown that the effects of *MAT* heterozygosity on cell survival following DNA damage reflect functions of both the Mat**a**l and Matα2 gene products, but were independent of *RME1,* a known downstream target of the Mat**a**1/Matα2 repressor. To date, the specific

target(s) of this repressor complex responsible for the effects of *MAT* on radioresistance have not been identified. In contrast to UV but similar to IR, *MAT* heterozygotes are more resistant than homozygotes to the cytotoxic effects of alkylating agent MMS *(50).* This is not surprising because both MMS and IR produce SSBs and DSBs.

3.3. Effects of MAT on UV-Induced and Spontaneous Homologous Recombination

It is not known how spontaneous recombination is initiated, but DSBs are often suggested as possible initiators. Spontaneous DSBs might arise at stalled replication forks *(69),* replication past SSBs (*see* Chapter 2 and ref. *24*), and in a cell cycle-independent manner from repair of oxidative or other forms of spontaneous DNA damage. A central role in vertebrate cells for RAD51-mediated recombination in replication restart at stalled or collapsed replication forks or in lesion bypass is suggested by the finding that RAD51 is essential for viability of higher eukaryotic cells, and that cells depleted for RAD51 accumulate chromosome and chromatid breaks *(48,77).* DSBs also may initiate UV-induced recombination, although much of this evidence is indirect (*see* Chapter 13).

Friis and Roman *(18)* were the first to show that *MAT* heterozygotes had higher homologous recombination frequencies than *MAT* homozygotes. In this study, *MAT* heterozygosity increased UV-induced allelic (homolog) recombination by three-fold. Similar *MAT* effects on UV-induced recombination were obtained in subsequent studies with allelic *(14,21)* and ectopic recombination substrates *(16).* As described for the radioprotective effects of *MAT,* enhanced recombination in *MAT* heterozygotes is clearly owing to *MAT,* and not just ploidy, because enhanced recombination is seen both in diploids and in haploids expressing both **a** and α *(16).* One concern of recombination studies is that *MAT* heterozygotes have the capacity to sporulate and some cells do so even on rich medium. In this small fraction of cells recombination would be increased to meiotic levels, and this could give an apparent increase in mitotic recombination. In most studies meiosis is thought to contribute little or not at all to the observed differences; this question could be answered by using *spo11* mutants, which fail to induce meiotic DSBs but are otherwise recombination-competent *(37).*

Three studies showed that spontaneous allelic recombination was higher in *MAT* heterozygotes than homozygotes *(15,21,36).* However, *MAT* genotype did not affect spontaneous sister chromatid recombination *(36),* suggesting that *MAT* heterozygosity may only enhance interactions between homologs. The lack of a *MAT* effect on sister chromatid recombination may be a consequence of the close, topologically constrained association of sister chromatids *(23,33),* such that *MAT* heterozygosity does not further stimulate these interactions. This view is consistent with the finding that sister chromatids are preferred recombinational repair templates in yeast *(36).* An alternative, but not mutually exclusive idea is that *MAT* genotype directly controls the frequency of spontaneous allelic recombination, perhaps by modulating the levels of recombination proteins. One such protein is Tid1p (Rdh54p), which is a homolog of Rad54p. Deletion of this protein reduces interchromosomal but not intrachromosomal homologous recombination *(3,40,75).* Tid1p plays a much more central role in meiosis, where it interacts with meiosis-specific strand-exchange protein, Dmc1p (a Rad51p homolog). Curiously, Tid1p is expressed at higher levels in **aa** and αα cells than in **a**α cells *(19).*

It is possible that the effects of *MAT* on homologous recombination and cell survival reflect specific effects on DSB repair, including recombinational repair. Although this idea is appealing in its simplicity, it will be very difficult to rule out other factors (e.g., changes in chromatin structure), because a large number of genes, many with unknown functions, are differentially expressed in the different mating types *(19,94).*

3.4. Recombination Between Ty Elements is Not Influenced by MAT

Studies of Ty recombination provide some interesting contrasts with other recombination systems. IR does not enhance Ty recombination involving direct repeat popouts, or conversions of a marked (target) Ty by any of the approx 30 unmarked Ty elements scattered throughout the genome, regardless of *MAT* status *(42).* Thus, *MAT* heterozygosity does not have global effects on homologous recombination. It is noteworthy that Ty elements are generally refractory to damage-induced recombination—even UV, a very potent recombinogen, has minimal stimulatory effects (and no *MAT* effects) on ectopic Ty recombination *(42).* In the same cells used to measure Ty recombination, ectopic recombination between 300 bp direct repeats at a non-Ty locus was monitored simultaneously, and modest enhancements with IR were observed. In agreement with Kadyk and Hartwell *(36)* but not Fasullo and Dave *(16), MAT* had no effect on the frequency of these non-Ty events *(42).* It is unclear why recombination in Ty elements is poorly enhanced by UV and IR, and not influenced by *MAT,* but it is clear that Ty elements are able to recombine, because DSBs introduced into a Ty element by HO endonuclease strongly enhance Ty recombination *(65).* Although not yet tested, it seems likely that the frequency of HO-induced Ty recombination events will be affected by *MAT* owing to *MAT* control of precise NHEJ (*see* Subheading 3.6.).

3.5. Effects of MAT on Ionizing Radiation- and HO-Induced Homologous Recombination

Although DSBs are potent initiators of homologous recombination (reviewed in refs. *63,64*), and IR is very effective at producing DSBs *(84),* recombination is only moderately enhanced by IR. This is because DSBs are most effective at stimulating recombination when they occur within or near a target gene, and each irradiated cell is likely to sustain considerable damage at other loci before a target gene is damaged in a significant fraction of a cell population. The effects of *MAT* on IR-induced recombination have been variable (Table 1, studies 1–4). *MAT* genotype had no effect on X-ray-induced recombination: unequal sister chromatid recombination between short (305 bp) direct repeats was not stimulated by X-rays in **aa** or **a**α cells, and both cells types showed the same increases in allelic recombination at each dose tested *(36).* In contrast, *MAT* heterozygosity increased by about two-fold the level of IR-induced ectopic recombination between duplicated regions of *his3* (~500 bp in length) located on different chromosomes; in this system selected His^+ recombinants reflect only reciprocal chromosome translocations *(17).* Interestingly, although *MAT* influenced IR-induced *his3* translocations in diploids, introduction of a plasmid carrying the opposite *MAT* allele had no effect on these same *his3* substrates in haploids *(17).* It was suggested that this ploidy difference reflected stimulation in diploids by damage sustained by homologous chromosomes not carrying the translocation substrates, by so-called triparental recombination events *(67);* such interactions are not possible in haploids. In contrast to the results

Table 1
***MAT* Effects on IR- and HO-Induced Recombination**

Study	Ploidy	Recombination substrate	Damaging agent	Recombination		Reference
				Stimulation	Increased by *MAT* heterozygosity?	
1	Diploid	Sister chromatid	Ionizing radiation	None	No	*(36)*
2	Diploid	Translocation	Ionizing radiation	Moderate	Yes	*(16)*
3	Haploid	Translocation	Ionizing radiation	Low	No	*(16)*
4	Diploid	Allelic	Ionizing radiation	Moderate	No	*(36)*
5	Diploid	Translocation	HO endonuclease	Strong	Yes	*(16)*
6	Haploid	Translocation	HO endonuclease	Strong	Yes	*(16)*
7	Diploid	Allelic	HO endonuclease	Strong	Yes	*(44)*
8	Diploid	Allelic	HO endonuclease	Strong	Yes	*(38)*

with IR, studies of HO-induced recombination show a much more consistent picture, with strong enhancements of ectopic (translocation) and allelic events, and marked effects of *MAT* heterozygosity (Table 1, studies 5–8). It is particularly interesting that damage-induced translocations show no effect of *MAT* heterozygosity with IR, but clear effects with HO endonuclease.

How can these disparate results be reconciled? To answer this, we need to draw on several lines of evidence. First, inactivation of NHEJ, by *yku70* or *yku80* knock-out, does not decrease radioresistance in yeast *(60).* This contrasts with marked radiosensitivity conferred by Ku mutations in mammalian cells (*see* Chapter 17 in Vol. 2). Furthermore, a *yku70 rad52* double mutant is only slightly more radiosensitive than a *rad52* single mutant *(76).* Thus, NHEJ is not involved to any great extent in the repair of IR damage in yeast. Second, *MAT* heterozygosity increases resistance to IR, an effect that is eliminated by mutations in recombinational repair genes (*RAD52* epistasis group). Together, these results indicate that homologous recombination is by far the most important repair pathway for IR damage, and that *MAT* heterozygosity increases radioresistance by enhancing recombinational repair, perhaps by enhancing pairing activity.

The magnitude of any effect of *MAT* on pairing is likely to depend on the intrinsic pairing properties of the recombination substrate under study. For example, sister chromatid recombination may not be enhanced by *MAT* heterozygosity (Table 1, study 1) because of the close association between sister chromatids, as discussed in Subheading 3.3. In contrast, the limited pairing between ectopic repeats on nonhomologous chromosomes may be enhanced by *MAT* heterozygosity (Table 1, study 2). However, because this effect was seen in diploids but not haploids (Table 1, compare studies 2 and 3), enhanced pairing may be limited to homologs (revealed as triparental recombination events). From this discussion, one would predict that IR-induced allelic interactions would be enhanced by *MAT* heterozygosity, but this was not the case (Table 1, study 4). This may reflect selection bias since a *MAT*-dependent increase in recombination events might go undetected if a large fraction of events involved co-conversion of the *leu1-1* and *leu1-12* alleles examined in this study (producing unselected homozygous *leu1-1/leu1-1* or *leu1-12/leu1-12* products). To understand the effects of *MAT* on HO-induced events and how these differ from IR-induced events, we need to consider the difference between precise and imprecise NHEJ (discussed in the next section), and the effects of *MAT* on NHEJ (discussed in Subheading 3.7.).

Finally, there is likely to be an inherent difference between the way nuclease-cleaved DNA engages in recombination compared to IR-broken molecules. One indication of this difference emerges from examining the effect of a single DSB on the viability of cells deleted for the *RAD52* recombination gene. Ho and Mortimer *(32)* reported that a single X-ray-induced DSB was dominant lethal in *rad52* haploids, diploids and even tetraploids, but this is clearly not the case in *rad52* diploids suffering an HO-induced DSB *(41,54).* IR-induced DSBs likely have glycol fragments attached to the phosphates and may be channeled into somewhat different recombination repair pathways.

3.6. Imprecise and Precise NHEJ in Yeast

Because DSB-repair in yeast by *RAD52*-dependent homologous recombination is so efficient, early strategies to detect NHEJ in yeast chromosomes employed *rad52*

mutants *(41,61),* or systems in which DSBs were introduced into regions for which no homologous repair template was available *(55,71,72).* The repair of HO endonuclease breaks and dicentric chromosome breaks by NHEJ results in similar junctions *(41).* These selective systems detected only imprecise NHEJ events, which were quite rare (typically <1% of homologous recombination levels). A nonselective assay was used to monitor imprecise NHEJ in recombination-competent cells (i.e., Rad^+ with DSBs introduced into a region of shared homology). Imprecise NHEJ of HO-induced DSBs was shown to occur at ~0.1% of homologous recombination levels *(38),* similar to *rad52* results. Thus, the efficiency of imprecise NHEJ is independent of Rad52p.

The identification of yeast homologs of mammalian NHEJ genes, Ku70 and Ku80 *(9,10,60),* and the use of a plasmid transformation-based rejoining assay *(9,10,60,83),* greatly facilitated studies of NHEJ in yeast. In particular, these assays indicated that nuclease DSBs in plasmid DNA are repaired very efficiently by precise NHEJ, and that this repair requires *YKU70, YKU80, LIF1,* and *LIG4,* and also involves *RAD50, XRS2, MRE11* (reviewed in ref. *13*).

It has been difficult to measure the efficiency of precise NHEJ of chromosomal DSBs because such events are not easily distinguished from "nonevents" (i.e., no DSB). However, the fact that yeast cells are able to survive expression of *Eco*RI, which has thousands of target sites per genome strongly suggested that precise NHEJ of nuclease DSBs in chromosomal DNA was quite efficient *(5,45,46).* A more quantitative measure of precise NHEJ of nuclease DSBs in chromosomal DNA was provided by Lee et al. *(44).* In this study, HO cleavage of *MAT* in haploid cells deleted for *HML* and *HMR* is largely lethal. However, when HO expression was limited to 1 h, 33% of cells survived compared to <1% survival of a *yku70* mutant. These results indicate that at least 33% of HO-induced chromosomal DSBs can be repaired by precise NHEJ. A different approach led to a similar conclusion in haploid cells, with at least 20% of HO DSBs in chromosomal DNA repaired by precise NHEJ (*see* Subheading 3.7.). Precise repair of nuclease-induced DSBs in mammalian chromosomes was recently reported *(49).*

3.7. Mating-Type Regulation of NHEJ and Homologous Recombination

About the same time that Ku was shown to mediate NHEJ in yeast, other lines of investigation revealed numerous connections between Ku and telomere structure/function (reviewed in refs. *27,52*). *yku 70* and *yku80* mutants were found to have shortened telomeres, and a similar phenotype was seen in *tel1* mutants *(9,66).* Genes near telomeres are silenced by binding of Sir2p, Sir3p, and Sir4p to telomere sequences; these proteins also silence *HML* and *HMR.* Extra DNA termini were found to relieve telomeric silencing *(87),* suggesting that one or more factors competed by DNA termini was involved in silencing, such as Ku, with its known end-binding function, or silencing proteins such as Sir, Rap1p, or Rif proteins. The involvement of Ku in telomere structure and function raised the possibility that other telomere-associated proteins (i.e., Sir2-4p and Tel1p) might be involved in NHEJ, and experiments appeared to confirm this possibility: NHEJ in yeast (assayed by plasmid end-rejoining) was reduced by 10- to 20-fold in *sir2, sir3* or *sir4* mutants, but not in a *tel1* mutant *(8,82).* Furthermore, *sir2, sir3,* and *sir4* mutants each displayed radiosensitivity phenotypes remarkably similar to *yku70* mutants, with increased sensitivity to IR revealed only in a *rad52* mutant background *(82).*

Initially these results were interpreted to mean that Sir proteins were directly involved in NHEJ. However, two recent studies showed that most or all of the *sir* effect on NHEJ is owing to *MAT* heterozygosity. In a study using transfected linear plasmids, deletion of all three mating-type loci suppressed the *sir*-dependent reduction in plasmid end-rejoining *(4).* In the second study *(44),* NHEJ was monitored for DSBs induced in vivo in plasmid and chromosomal DNA. In a *MAT***a** haploid lacking *HML*α, *sir* mutants do not yield an **a**α state and *sir* mutants were shown to have wild-type levels of plasmid retention (~50%) when DSBs were created by nucleases or by mechanical breakage of a dicentric plasmid. In contrast, plasmid retention was strongly reduced in *yku70* mutants. Similar results were obtained for chromosomal DSBs. In haploid wild-type cells deleted for *HML* and *HMR,* continuous expression of HO (which cleaves *MAT*α) was largely lethal because homologous recombination is blocked (~0.1% survivors). Survivors arise by NHEJ that yields deletions or insertions in *MAT*α (preventing further cleavage by HO). Survival is reduced an additional 3-orders of magnitude in *yku70,* demonstrating the importance of Ku for these imprecise NHEJ events, but *sir2* had only a modest two- to three-fold effect on survival. Thus, the effect of *sir2* on NHEJ is largely owing to *MAT* heterozygosity. The chromosomal assay (but not the plasmid rejoining assay) revealed that *sir3* and *sir4* mutants had two-fold lower levels of NHEJ regardless of *MAT* genotype (*sir2* had a smaller effect) *(44).* These results suggest that Sir proteins also have a direct role in end-joining, and this has been supported by cytological and chromatin immunoprecipitation experiments, indicating that both Sir and Ku proteins translocate from telomeres to sites of DSB damage *(56,57,59).* It is not known how *MAT* controls NHEJ. Expression levels of several genes involved in NHEJ were measured in *MAT* homozygous and heterozygous strains, including *YKU70, YKU80, LIG4, XRS2,* and *MRE11,* but none were differentially expressed *(4);* however, the use of microarray technology has revealed other genes that are downregulated by mating type and that might be involved in NHEJ *(19).*

Because downregulation of NHEJ by *MAT* heterozygosity serves to increase HO-induced homologous recombination, one would predict that elimination of NHEJ by *yku70* mutation would similarly increase recombination. In agreement with this prediction, recombination induced by HO endonuclease cleavage of a chromosomal *ura3* direct repeat substrate in haploid cells was 1.25-fold higher in a *yku70* mutant than wild-type *(38).* This result suggests that at least 20% of HO-induced DSBs are repaired by precise NHEJ in wild-type haploid cells. The opposite result was seen for HO-induced mating-type switching, with *yku70* mutants displaying three-fold lower levels than wild-type; *yku70* mutants also had 10- to 40-fold lower levels of spontaneous allelic recombination *(53).* However, in this study the W303-derived cells likely carried a *rad5* mutation, and the reduced recombination in *yku70* may reflect a genetic interaction with *rad5.* This idea is supported by a study showing that Rad5 is involved in channeling repair from NHEJ to gene conversion *(1).* In another study it was found that *yku70* mutants have wild-type levels of meiotic recombination *(83).* The lack of an effect of *yku70* on meiotic recombination is consistent with a reduced (or minimal) role for NHEJ in **a**α cells (the required *MAT* genotype for meiosis). Interestingly, Ku is reduced in abundance in mammalian meiotic cells *(22);* this reduction may be required to minimize NHEJ in higher eukaryotic cells during meiosis, thereby promoting homologous recombination.

It is now clear that the NHEJ pathway in yeast is ineffective in the repair of IR damage, very effective at precise rejoining of nuclease-induced (ligatable) DSBs, upregulated in cells homozygous at *MAT,* and downregulated in cells heterozygous at *MAT.* In this light, the differential effects of *MAT* heterozygosity on IR and HO-induced homologous recombination shown in Table 1 can be readily explained. Both homologous recombination and NHEJ compete for the repair of HO-induced DSBs. In *MAT* homozygous strains, NHEJ is upregulated and this reduces the number of DSBs processed by homologous recombination. In *MAT* heterozygous strains, NHEJ is downregulated and homologous recombination increases because essentially all DSBs are shunted to the homologous recombination pathway. Thus, *MAT* indirectly influences nuclease-induced recombination by modulating NHEJ. As described in Subheadings 3.1. and 3.5., *MAT* directly influences IR-induced recombination. There is evidence that *MAT* heterozygosity also enhances nuclease-induced homologous recombination, independently of its effects on NHEJ. A study of HO-induced allelic recombination vs chromosome loss showed that recombination was nearly 100% efficient in an **a**α diploid but only 82% efficient in an Δα diploid *(44).* Similarly, even when NHEJ is inactivated by *yku70* mutation, HO-induced allelic recombination was higher in **a**α cells than in **aa** cells *(38),* although an alternative explanation for this result is that DNA ends in *yku70* mutants might be altered (i.e., longer single-stranded tails) and might be better substrates for a *MAT*-regulated protein involved in allelic recombination, such as Rdh54p *(40,75)* which is expressed at five-fold higher levels in **aa** cells than in **a**α cells *(19).*

It has been proposed that upregulation of NHEJ in *MAT* homozygous strains reflects an increased need for this type of repair in G1 haploid cells as these lack homologous repair templates *(4,44).* This hypothesis suggests that homologous recombination is the preferred repair mode when available. Recombination has been suggested to confer both short- and long-term (evolutionary) advantages. Recombination is an essential feature of meiosis, with crossovers required for accurate chromosome segregation *(12),* and both meiosis and *MAT* heterozygosity confer competitive growth advantages in mixed culture experiments *(7).* An alternative, but not mutually exclusive hypothesis derives from considerations of the types of DSBs formed most frequently in natural settings, including those that result from replication, mechanical stress (i.e., in dicentric chromosomes), endogenous nucleases (including failed type II topoisomerase reactions), or other DNA repair processes such as nucleotide-excision repair operating on closely opposed pyrimidine dimers. For perhaps many of these types of DSBs, even if NHEJ were possible, repair by this pathway might not be precise. It may be that high-fidelity repair by homologous recombination, despite the potential for gross chromosomal rearrangement, provides for optimal genome stability.

4. MATING-TYPE REGULATION OF DONOR PREFERENCE DURING HO-INDUCED SWITCHING OF THE *MAT* LOCUS

One of the most fascinating aspects of mating-type control of recombination is that during HO-induced switching of the *MAT* locus, *MAT***a** and *MAT*α cells show a strong preference to interact with different silent mating type donors, *HML* and *HMR (39).* Thus, a *MAT***a** cell will recombine with *HML* four to five times more often than with *HMR.* This preference persists even if both *HML* and *HMR* carry the same Y**a** or Yα region and even if *HML* is replaced with a cloned *HMR* segment, indicating that chro-

mosomal position dictates this preference *(85,92).* In fact, recombination is regulated along the entire 113 kbp left arm of chromosome III, as an *HML* or *HMR* sequence placed at several other locations along this arm is preferentially selected in *MAT***a** cells and preferentially excluded in *MAT*α cells *(90,91,93).* Moreover, the activation of the left arm for recombination (or rather, the repression of the left arm in *MAT*α cells) is not specific to HO-induced events involving silent donor sequences; a *leu2-R* allele inserted into a complete deletion of *HML* and its surrounding silencer sequences shows higher levels of recombination with a *leu2-K* allele in *MAT***a** vs *MAT*α cells, even when the *leu2-K* allele is present on another chromosome. Evidence suggests that in *MAT*α cells the left arm is somehow made inaccessible for recombination, as a *MAT*α cell carrying *HML*α but deleted for *HMR* frequently dies rather than completing recombination using the (excluded) left-arm donor *(90).* Whatever accounts for this exclusion, it is not a general heterochromatinization or condensation of the left arm, as there is no evident change in the expression of genes along the arm in *MAT***a** versus *MAT*α cells, as measured by microarrays *(19).*

A major breakthrough in understanding the mechanism of donor preference was the identification of a small *cis*-acting sequence that controls donor preference, located about 17 kbp centromere-proximal from *HML (91).* This Recombination Enhancer (RE) was "whittled down" to a 244 bp sequence that retains much of the activity *(89).* Further dissection has indicated that three subregions comprising only 137 bp has activity (K. Sun and J.E.H., unpublished). When RE is deleted in *MAT***a** cells, the left arm adopts the "cold" phenotype found in *MAT*α cells. Further work showed that the inactivation of RE was accomplished by the binding of the Matα2p-Mcm1p co-repressor, which is known to regulate **a**-specific genes *(80,81,89).* Under these conditions, the entire 2.5 kbp noncoding region in which RE resides is occupied by highly positioned nucleosomes. Activation of RE in *MAT***a** cells depends on the Mcmlp protein, which also activates transcription of **a**-specific genes, although the roles of other proteins that presumably bind to the other segments of RE have not been identified. How RE acts to antagonize the coldness of the "default" state remains a mystery. One general idea is that the left arm is sequestered—perhaps bound to the nuclear envelope—in *MAT*α cells, thus preventing easy diffusion to participate in recombination, but is somehow freed in *MAT***a** cells.

5. CONCLUDING REMARKS

There are several key questions that remain about *MAT* regulation of DNA repair and recombination. A major gap in our knowledge concerns the mechanism by which *MAT* regulates NHEJ. It is unclear whether *MAT* effects on NHEJ and homologous recombination are genetically separable, and whether they reflect pleiotropic effects of a single gene or set of genes. Microarray analysis of mRNA levels in cells with various *MAT* genotypes has provided a huge number of candidate targets for further investigation *(19).* However, *MAT* control of NHEJ may reflect post-transcriptional regulation and may not be revealed by analysis of mRNA levels. The mechanism of action of RE also remains a mystery. A molecular description of the RE regulatory system should provide important insights into mechanisms of recombination suppression in yeast and higher eukaryotes as well.

It is now apparent that organisms regulate recombination and DNA repair in many ways, during different stages of the cell cycle, during different developmental and life-

cycle stages, and in response to environmental insults. Even within a cell, recombination is differentially regulated at various chromosomal domains. The *MAT* locus represents a particularly interesting case because it has strong regulatory effects on a variety of recombination and repair processes, and *MAT* expression is itself regulated by a recombinational repair event. Metazoans also employ recombinational repair to control gene expression, exemplified by gene rearrangements during development of the immune system (*see* Chapter 11). It is likely that higher eukaryotes employ a variety of mechanisms by which cellular recombination and repair capacities are regulated during growth and development. The p53 protein provides one such example: embryonic stem cells and differentiated somatic cells differ markedly in their p53 responses to DNA damage, including altered translocation of p53 to the nuclease, altered checkpoint function, and altered apoptotic response *(2).* Wild-type p53 functions to repress homologous recombination *(6,58,78,86)* and embryonic stem cells display enhanced homologous recombination (as measured by gene-targeting efficiency). p53 repression of homologous recombination may be transiently relieved in embryonic stem cells to enhance recombinational repair of endogenous lesions during the characteristic rapid growth of these cells. The elucidation of this and other networks that modulate DNA repair and recombination in different cell types and developmental states will provide many interesting challenges for the future.

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6

DNA End-Processing and Heteroduplex DNA Formation During Recombinational Repair of DNA Double-Strand Breaks

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1. INTRODUCTION

1.1. Pathways of DNA Double-Strand Break Repair in Eukaryotes

Two enzymatic mechanisms, DNA end-joining and homologous recombination, operate in eukaryotic cells to repair DNA double-strand breaks (DSB) induced by ionizing radiation and by other agents. The recombinational repair pathway relies on an intact DNA homolog to direct the healing of the DNA break, and is designed to restore the original configuration of the injured chromosome. In contrast, the DNA end-joining process has no requirement for a DNA homolog and often results in gain or loss of genetic information, and at times chromosomal rearrangements and translocations. There is emerging evidence that DNA end-joining and homologous recombination are differentially required at specific stages of the cell cycle, with the former appearing to be the more critical mechanism in the G1 phase and the latter taking on a prominent role in late S and G2 when a sister chromatid becomes available to direct the repair process *(113)*. In this article, we will provide an account of what is currently known about homologous recombination and its DNA repair role.

1.1. Repair by Homologous Recombination

This is an excellent time to review DNA DSB repair by homologous recombination. A great many years of exhaustive genetic studies in *Saccharomyces cerevisiae* have served up conceptual and genetic frameworks for appreciating the intricacies of recombination processes and, most importantly, have paved the path for mechanistic dissection of these processes utilizing biochemical and cell biological approaches. The recent identification of genes in higher eukaryotes that are clear analogs of the yeast recombination/repair genes further indicates that studies in *S. cerevisiae* will be informative with respect to these processes in mammals. Nonetheless, although there is little doubt that the fundamental mechanisms of recombination processes have been conserved from yeast to humans, these processes in higher eukaryotes are apparently subject to additional layers of control, some of which are dependent on known tumor suppressors

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including the gene mutated in ataxia talengiectasia *(ATM),* the breast tumor-suppressor genes *BRCA1* and *BRCA2,* the gene mutated in Nijmegen breakage syndrome *(NBS),* and almost certainly with additional as yet unknown genes. Although the precise manner in which these tumor-suppressor genes modulate the DNA DSB repair process remains to be elucidated, these observations, coupled with the known role of recombination genes in helping maintain genomic stability, have provided tantalizing clues as well as tangible evidence that the recombination machinery is intimately linked to cancer suppression in mammals.

1.3. The RAD52 Epistasis Group Required for Recombination Processes

Genetic experiments in yeast have indicated that aside from its DNA repair role, the recombination machinery functions to establish stable interactions between chromosomal homologs during the meiotic prophase, and as such, is indispensable for the proper disjunction of homologs in meiosis I *(91).* In *S. cerevisiae,* the two meiotic divisions, if successfully completed, yield four haploid spores. The requirement for homologous recombination in meiotic chromosome homeostasis is aptly manifest as a nearly complete failure of recombination defective mutants to undergo sporulation, and low viability of the few spores that are formed *(91).* In addition, the process of mating-type switching in *S. cerevisiae,* which is initiated by a site-specific DNA DSB at the mating-type locus made by the HO endonuclease, is also dependent on the recombination functions for its completion *(77).* Much of the present knowledge about recombination in *S. cerevisiae* has in fact been garnered from studies on meiotic recombination, HO-induced mating-type switching, and from other model systems involving HO-induced recombination processes.

A number of genes, namely, *RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, RDH54/TID1, MRE11,* and *XRS2,* collectively known as the *RAD52* epistasis group, are required for homologous recombination processes *(81,35,4,55,102).* Aside from the *RAD52* epistasis group, the meiotic recombination program is dependent on a plethora of meiosis-specific factors, some of which are involved in the introduction of DNA DSB at various recombination "hotspots" along each of the chromosomes (*see 54* for a discussion), while others function in checkpoint mechanisms coordinating recombination and cell-cycle progression *(64,123),* and the *RAD51* homolog *DMC1 (10,11).*

1.4. DNA Double-Strand Breaks as Progenitor of Homologous Recombination

Because DNA DSBs are potentially lethal lesions, intuitively, it is somewhat curious as to why programmed recombination processes are initiated via the formation of these breaks. This curiosity aside, it is a very well-established fact that mating-type switching and meiotic recombination in *S. cerevisiae* are triggered by the introduction of DNA DSBs at discrete chromosomal loci. Genetic experiments have provided evidence that the meiotic DNA DSBs are made by a multicomponent complex comprising Rad50, Mre11, Xrs2, Spo11, and other proteins. Interestingly, Spo11 shows significant sequence homology to an archaebacterial type II topoisomerase, and mutation of tyrosine 135 in Spo11, which is equivalent to the active site tyrosine in other topoisomerases, abolishes its biological function *(9).* These observations, together with the fact that Spo11 can be found associated with the 5′ termini of the ends of the DNA breaks *(54),* lend credence to the suggestion that Spo11 protein is the catalytic subunit of the protein machinery that makes DNA DSBs during meiotic prophase *(9,54).*

Importantly, recent studies have identified structural and functional homologs of Spo11 in other eukaryotes. Villenenue and colleagues *(25)* have reported that inactivation of the Spo11 homolog in *Caenorhabditis elegans* results in no overt somatic abnormalities but a constellation of phenotypes consistent with a defect in meiotic chromosome segregation. As assessed by cytological and genetic methods, meiotic crossovers are abolished in worms lacking the Spo11 protein. Mutant *spo11* worms produce a normal number of oocytes, but the vast majority die as embryos. Of the few mutant embryos that survive to adulthood, roughly half are male (XO). As *C. elegans* is normally hermaphroditic possessing two X chromosomes, the preponderance of XO males is an indication of nondisjunction of the X chromosomes. The suggestion that the meiotic defects in the *spo11* mutant animals are owing to an inability to form DNA DSBs is reinforced by the finding that meiotic crossovers and embryonic viability can be restored by treatment of the *spo11* mutant worms with γ-rays. Taken together, the results strongly suggest that like its yeast counterpart, *C. elegans* Spo11 is also required for meiotic recombination, probably for the introduction of meiosis-specific DNA DSBs *(25).* The *mei-W68* gene in the fruitfly *Drosophila melanogaster* is required for meiotic gene conversion and crossing-over, suggesting that it is involved in the initiation of meiotic recombination. The *mei-W68* gene encodes a homolog of Spo11, a finding that provides further support to the notion that Spo11-mediated formation of DSBs is indispensable for the initiation of meiotic recombination among eukaryotes *(66).*

1.5. Current Model for Recombination Induced by DNA Double-Strand Breaks

The DNA DSB repair model for recombination presented by Sun et al. *(105)* provides a conceptual framework for the discussions that follow. In this model (Fig. 1), the ends of the DNA breaks are processed to yield long 3′ single-stranded overhangs, which serve as the substrate for the recruitment of recombination factors. Nucleation of the recombination factors onto the ssDNA tails renders them recombinogenic, leading to a search for an intact DNA homolog, a homologous chromosome or a sister chromatid, and invasion of the DNA homolog to yield heteroduplex DNA. It is important to emphasize that the 3′ ssDNA tails depicted in this model have been detected in numerous studies *(16,102,105),* as have the double Holliday junctions been observed *(95).* In light of this model, we review what is known about the enzymatic reactions that are needed to initiate and complete the recombination process.

2. THE DNA DSB REPAIR REACTION

2.1. DNA End Processing

2.1.1. The Rad50, Mre11, and Xrs2/NBS1 Proteins

RAD50, MRE11, and *XRS2* encoded products are involved in the nucleolytic end-processing reaction that yields the 3′ ssDNA tails (Fig. 1). Results from yeast two-hybrid studies have suggested that these proteins interact with one another *(49).* Rad50 is a member of the *s*tructural *m*aintenance of *c*hromosome (SMC) protein family, possessing coiled coil domains as well as ATP binding motifs. Mre11 exhibits homology to phosphodiesterases, while Xrs2 has a putative forkhead-associated (FHA) domain, which may be involved in protein-protein interactions. Human NBS1, the presumed functional equivalent of yeast Xrs2, possesses an FHA domain and a breast cancer car-

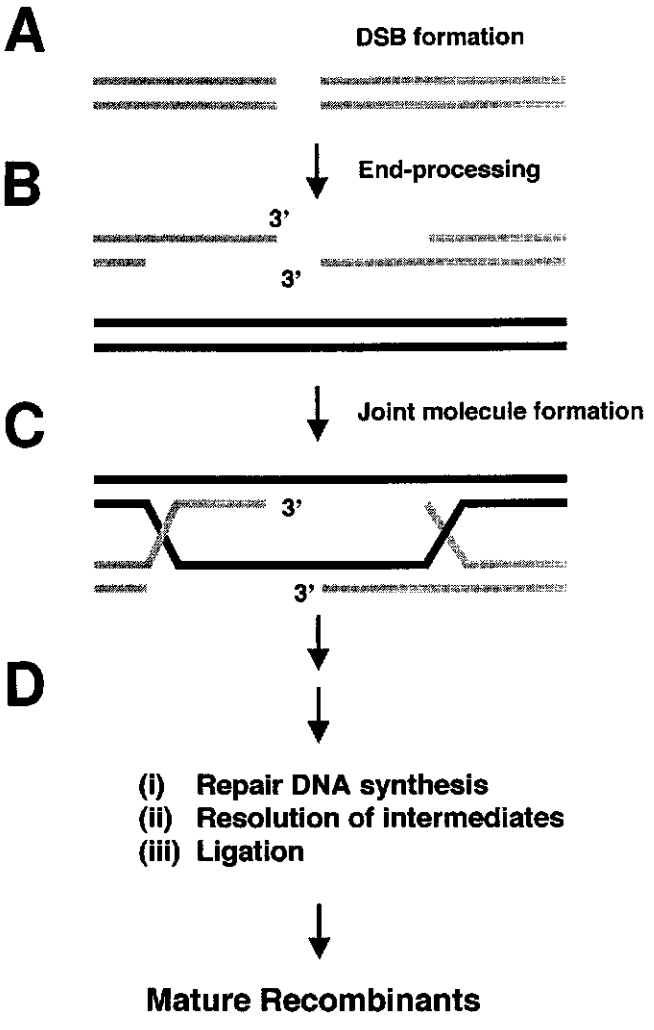

Fig. 1. DSB repair model for recombination. The repair reaction begins with the nucleolytic processing of the break to create a 3′ ssDNA tail for the nucleation of Rad51 and other recombination factors to form a nucleoprotein complex, which has the ability to search for a DNA homolog and mediates DNA strand invasion to form heteroduplex DNA. Repair DNA synthesis serves to replace the genetic information eliminated during end-processing, and resolution of the Holliday junctions followed by DNA ligation then yield mature recombinants.

boxy-terminal (BRCT) domain, which are believed to be involved in protein-protein interactions *(26,32,82).* Human Rad50 and Mre11 have been identified recently *(26,82).* The Rad50 and Mre11 proteins are structurally related to the *Escherichia coli* SbcC and SbcD proteins, respectively *(96).* SbcC and SbcD combine to form a complex that has ATP-independent ssDNA endonuclease and ATP-dependent dsDNA exonuclease activities, as well as an ability to open DNA hairpins *(21).* Consistent with the homology of Rad50 and Mre11 to the SbcC/SbcD nuclease complex, yeast and human Mre11 as well as the protein complex consisting of human Mre11 and associated proteins also possess endonuclease and exonuclease activities (*see* below).

Immunoprecipitation studies indicated that Rad50, Mre11, and three additional protein species having molecular sizes of about 95 kDa, 200 kDa, and 400 kDa are associated in human cell extracts *(26).* Partial purification of the protein complex containing Rad50, Mre11, and associated proteins and peptide microsequencing helped identify the p95 species as the product of the gene mutated in Nijmegen breakage syndrome, a disease characterized by cellular sensitivity to DNA damaging agents, chromosomal fragility, and a high incidence of malignancies. The p95 species is now also referred to as NBS1 or nibrin. The 200 kDa species has been identified as fatty-acid synthase, whose association with the Mre11/Rad50/p95 was judged to be fortuitous *(17),* whereas the identity of the 400 kDa species is currently unknown. It is very likely that NBS1, which shows significant homology to yeast Xrs2 at the amino-terminus, represents the functional analog of Xrs2 in recombination and repair processes *(17,121).*

The biochemical properties of human Mre11 and the protein complex comprising human Rad50, Mre11, and NBS1 are well-characterized. Paull and Gellert *(78)* overexpressed the hMre11 protein in insect cells and purified it to near homogeneity. hMre11 possesses an exonuclease activity that removes mononucleotides from the 3′ termini of dsDNA. Coexpression of hMre11 and hRad50 in insect cells gives a stoichiometric hRad50/hMre11 complex that has four times the exonuclease activity of hMre11 alone. Because hRad50 is not expected to have any nuclease activity, the enhancement of nuclease function seen in the hMre11/hRad50 complex is likely owing to stimulation of hMre11 nuclease activity by hRad50. Whether hRad50 stimulates the hMre11 exonuclease activity by enhancing end recognition or processivity remains to be determined. Like the SbcC/SbcD nuclease complex *(21),* hMre11 also cleaves DNA hairpins *(78).* A more recent study from the Gellert laboratory shows that NBS1 complexes with hMre11 and with hRad50/hMre11. In the presence of ATP, NBS1 modulates the nuclease activities of hMre11 on hairpin substrates, enables hRad50/hMre11 to cleave a 3′ ssDNA overhang, and confers an ability to hRad50/hMre11 for the localized unwinding of duplex DNA *(79).* In addition, it was demonstrated by mutagenesis and biochemical means that hRad50 plays a critical role in the ATP-stimulated endonucleolytic and DNA unwinding activities of the hRad50/hMre11/NBS1 complex. hRPA and the hKu heterodimer, two abundant nuclear DNA binding factors, are found to compete with the hRad50/hMre11/NBS1 complex for DNA substrate sites. Although NBS1 may serve to relay the detection of DNA damage to the cell-cycle checkpoint machinery *(97),* the results of Paull and Gellert *(79)* have demonstrated dramatic effects of NBS1 on the biochemical properties of hRad50/hMre11.

In a parallel study, the hRad50/hMre11/NBS1 complex was purified to near homogeneity from nuclear extracts of Raji cells. However, the 200 kDa species (fatty-acid synthase) and the 400 kDa species did not copurify with the hRad50/hMre11/NBS1 complex *(118).* The purified hRad50/hMre11/NBS1 complex digests covalently closed single-stranded DNA circles and mcks supercoiled DNA. Quantification of the endonuclease activity of the hRad50/hMre11/NBS1 complex on single-stranded vs supercoiled DNA revealed that it is highly specific for single-stranded DNA. It was also shown that the endonucleolytic reaction products have 3′ hydroxyl and 5′ phosphate termini, and that the hRad50/hMre11/NBS1 complex possesses a 3′ to 5′ exonuclease activity *(118).*

Yeast Mre11 is also associated with Rad50 and Xrs2 in a complex *(49,120),* and purified yMre11 also possesses a ssDNA specific endonuclease activity *(34,71,120)* and a 3′ to 5′ exonuclease activity on dsDNA *(34,71,120)* and on ssDNA as well *(120).*

2.1.2. A Model for DNA DSB End-Processing

Extensive genetic studies in *S. cerevisiae* have indicated that the ends of DNA DSBs are processed in an apparent exonucleolytic fashion to yield 3′ ssDNA tails (Fig. 1). One can contemplate at least two possible mechanisms for accomplishing the end-processing reaction. One scenario would involve a classical 5′ to 3′ exonuclease activity that engages the DNA end and then removes nucleotides in reiterative fashion from the DNA strand that contains the 5′ terminus. The other, somewhat more elaborate scheme, would entail the cooperation between an endonucleolytic activity and a helicase function, unwinding the duplex from the extremity to create a ssDNA region acted on by the endonucleolytic function. The two possible mechanisms of end-processing may not be mutually exclusive, as a nuclease/helicase complex may possess all the activities required for mediating the end-processing reaction via both routes, or alternatively, an exonuclease and an endonuclease/helicase complex may independently provide end-processing functions in parallel reactions.

Given that the Mre11-associated protein complex is apparently devoid of a significant 5′ to 3′ exonuclease activity *(34,71,78,118),* the most pertinent query then becomes: How may the Mre11 nuclease activities be utilized for the end-processing reaction? One plausible scenario is that the 3′ to 5′ exonuclease activity, at least under some circumstances, makes a short 5′ single-stranded overhang for the loading of a DNA helicase, or one of a number of alternate helicases, to initiate DNA unwinding for creating a branched DNA structure for the Mre11 endonuclease function to act, as depicted in Fig. 2. We favor this hypothetical model of DSB end-processing for two reasons. First, it reconciles the polarity of DNA end-processing observed in vivo with the known biochemical properties of purified Mre11 and the Mre11/Rad50/NBS1 complex. Second, this model shares major features with the known mechanism of the DNA end-processing complex RecBCD during recombination processes in *E. coli,* which creates a recombinogenic 3′ ssDNA tail via DNA unwinding and internal scission of the unwound, 5′ overhanging DNA strand (reviewed in *57).* The validation of this working model (Fig. 2) will require the identification of the putative DNA helicase(s) that physically and functionally interacts with the Mre11 protein complex.

During the processing of meiotic DSBs, it is believed that the Mre11 endonuclease activity removes Spo11 that remains covalently attached to the 5′ termini of the DNA ends. In this case, the result of Spo11 removal may be the generation of a short 3′ overhang to be utilized for the loading of a DNA helicase to initiate DNA strand separation *(71,120).*

2.1.3. Multifunctional Nature of the Mre11-Associated Protein Complex

As mentioned earlier, in addition to mediating the DNA end processing reaction, the Rad50/Mre11/Xrs2 complex, in conjunction with Spo11 and other factors, is also required for the formation of DNA DSBs at the initiation sites of meiotic recombination (*see 54* for a discussion). Subsequent to the formation of meiotic DSBs, it is believed that Spo11 remains covalently attached to the 5′ termini of the DNA ends, and that the removal of the covalently conjugated Spo11 is mediated by the Mre11 endonucleolytic activity *(77).* Genetic studies *(13,70,93)* have also indicated a role for Rad50, Mre11, and Xrs2 in the DNA end-joining pathway of DSB repair as well. Remarkably, these three proteins have also been linked to the maintenance of telomere length *(13,77).* In mediating biological functions distinct from DNA DSB end-processing, Mre11 and

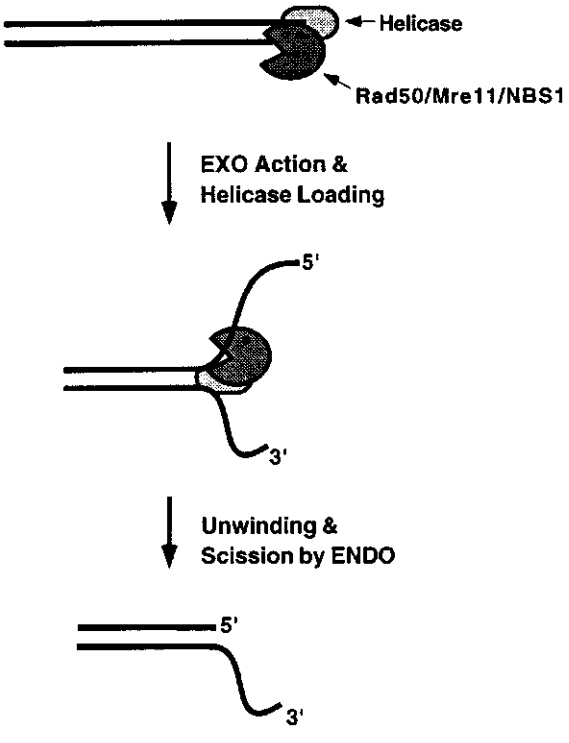

Fig. 2. A hypothetical model for DSB end-processing. The first step of DNA end-processing is postulated to be the creation of a short 5′ ssDNA overhang by the 3′ to 5′ exonuclease activity of the Rad50/Mre11/NBS1 complex. This is followed by the loading of a DNA helicase, DNA strand separation by the helicase, and endonucleolytic cleavage of the 5′ overhanging DNA strand to generate the 3′ ssDNA tail. This model may not apply in the case of the processing of DSBs generated during meiosis (*see* text for details).

associated proteins may provide a molecular scaffold for the recruitment of additional protein factors and the assembly of the appropriate protein complexes. This suggestion is supported by the observation that some the DNA end-joining events affected in *rad50, mre11,* and *xrs2* mutants do not actually require nucleolytic end-processing *(13).* In fact, inactivation of the Mre11 nuclease function has no discernible effect on end-joining and telomere-length maintenance *(71).* An intriguing genetic interaction between the Mre11/Rad50/Xrs2 complex and the Srs2 DNA helicase has been observed by Klein and colleagues; haploid *srs2 rad50, srs2 xrs2,* and *srs2 mre11* mutants have a severe growth deficiency and the mutant diploids are inviable. The precise reason for the synthetic growth deficiency and inviability remains to be elucidated, but it appears to be unrelated to the telomere maintenance function of Rad50/Mre11/Xrs2 *(56).*

3. HETERODUPLEX DNA FORMATION

Genetic exchange involves the formation of a heteroduplex DNA intermediate between the recombining homologous chromosomes (Fig. 1; *see* 112 for a discussion). Substantial genetic and biochemical evidence has implicated the *RAD51, RAD52, RAD54, RAD55, RAD57,* and *TID1/RDH54* genes in heteroduplex DNA formation;

Table 1
Factors that Function in Heteroduplex DNA Formation

Yeast factor	Known role	Human equivalent	Prokaryotic equivalent
Rad51	Recombinase	hRad51	RecA in *E. coli* UvsX in T4
Rad52	Mediator	hRad52	RecO/RecR in *E. coli* UvsY in T4
Rad55/Rad57	Mediator	XRCC2, XRCC3, Rad51B, Rad51C, and Rad51D[a]	RecO/RecR and UvsY
Rad54	Synaptic factor	hRad54	Not known
Rdh54	Synaptic factor[a]	Not yet identified	Not known
Rad59	Currently unknown	Not yet identified	Not known
Dmc1	Recombinase[b]	hDmc1	RecA and UvsX

[a] These proteins exhibit homology to the hRad51. Based on the paradigm established with yeast Rad55/Rad57 complex *(109),* we suspect that some or all of these human factors may also function as mediators with hRad51. As for yeast Rdh54, there is currently a paucity of information concerning its biochemical function. We tentatively assign a synaptic function to Rdh54, based on its similarity to Rad54 and on genetic observations that the two appear to play somewhat overlapping roles in recombination and repair.

[b] Based on studies with the human Dmc1 protein *(59).*

whether *RAD59* also has a role remains to be established. The basic mechanism for heteroduplex DNA formation is very likely conserved among eukaryotes, as structural/functional homologs of the yeast Rad51, Rad52, and Rad54 proteins have been identified in humans (*see 51* for a discussion), and characterization of the human proteins *(7,8,39,89,111)* revealed functional properties similar to those of their yeast counterparts *(73,74,83,100,101,103,104,106,107,110).* The recombination factors that function in heteroduplex DNA formation are listed in Table 1.

3.1. Role of Various Recombination Factors in Heteroduplex DNA Formation

The enzymatic process responsible for creating heteroduplex DNA during recombination is referred to as homologous DNA pairing and strand exchange. The family of proteins that mediate homologous DNA pairing and strand exchange, including *E. coli* RecA, bacteriophage T4 UvsX, and Rad51 from yeast and humans, are called "recombinases." Based on biochemical studies of prokaryotic and eukaryotic recombinases, some salient features concerning the activities of these enzymes and the homologous pairing and strand exchange reaction have emerged, as highlighted later.

3.1.1. The Rad51 Recombinase

The *RAD51* encoded product from both yeast and humans possesses homologous DNA pairing and strand-exchange activities *(7,39,103,106).* Biochemical studies have strongly suggested that, like the prokaryotic recombinases, the Rad51-mediated homologous DNA pairing and strand exchange reaction proceeds via two distinct stages, termed presynaptic and synaptic phases (reviewed in *57).*

3.1.2. The Presynaptic Phase

In the presynaptic phase, Rad51 polymerizes on ssDNA to form a right-handed protein filament that has a highly regular pitch (about 95 angstroms) and in which the DNA

is held in an extended conformation (axial rise of about 5 angstroms per base or base pair as compared to 3.4 angstroms per base pair for B form duplex DNA). The Rad51-DNA nucleoprotein filament is almost identical in overall dimensions and appearance to the equivalent filament of RecA protein *(75,107).* Biochemical studies have indicated that formation of heteroduplex DNA with the incoming duplex DNA partner occurs within the confines of this nucleoprotein filament *(107).* Although Rad51 possesses a DNA-dependent ATPase activity, the formation of the presynaptic nucleoprotein filament requires only ATP binding *(108),* as for RecA *(57).* The assembly of the Rad51 presynaptic filament is stimulated by the heterotrimeric ssDNA binding factor RPA, when RPA is added to the in vitro reactions after Rad51 has been allowed to nucleate onto the ssDNA *(103,109).* Paradoxically, when added to the ssDNA before or together with Rad51, RPA interferes with the assembly of the presynaptic filament, leading to an inhibition of heteroduplex DNA formation *(109).* Specific protein factors, termed mediators *(51,110),* have been identified that function to facilitate the assembly of the Rad51 filament when RPA is competing for DNA binding sites with Rad51. These mediators, Rad52 protein and the Rad55/Rad57 complex (*see* below), are functionally equivalent to *E. coli* RecO/RecR complex *(119)* and T4 UvsY *(40,46).*

3.1.3. The Synaptic Phase

In this reaction phase, the Rad51-ssDNA nucleoprotein filament conducts a search for DNA homology, resulting in the synapsis or pairing with the homolog, and formation of heteroduplex DNA with the homolog. Concerning the mechanism of the DNA homology search and pairing process, some clues have been gleaned from studies conducted with the RecA-ssDNA nucleoprotein filament. In this case, the nucleoprotein filament contains a second DNA binding site that accommodates the incoming duplex DNA molecule. Following the incorporation of the duplex molecule into the RecA-ssDNA nucleoprotein filament through nonhomologous contacts, homology is found, and alignment of the two recombining molecules is established through a series of transient, short heteroduplex joints called "paranemic" joints. When a free end is present either in the ssDNA or the dsDNA molecule, intertwining of the ssDNA strand with the complementary strand in the duplex partner occurs, resulting in the formation of a stable joint molecule called a "plectonemic" joint. Subsequent to the formation of the nascent plectonemic joint molecule, unidirectional branch migration, or DNA strand exchange, leads to the extension of the heteroduplex joint *(2,14,23,57,88).* It is likely that this paradigm (Fig. 3) will be applicable to Rad51 as well. In yeast, the rate of homologous DNA pairing by Rad51 is greatly accelerated by Rad54 (*see* Subheading 3.3.1.).

3.2. Mediators

As described earlier, RPA is required in the synaptic phase of the homologous pairing and strand exchange reaction. RPA is believed to remove the secondary structure in ssDNA that would otherwise impede the Rad51 filament assembly process *(103,107).* The stimulatory effect of RPA is seen most clearly when it is added to the reaction after Rad51 has already nucleated onto ssDNA. If RPA is added before or with Rad51, a pronounced inhibition of pairing and strand exchange ensues *(109).* This and other observations *(103,109)* have led to the deduction that RPA, in addition to its stimulatory role, can also compete with Rad51 for binding sites on the ssDNA substrate *(103,109).* The

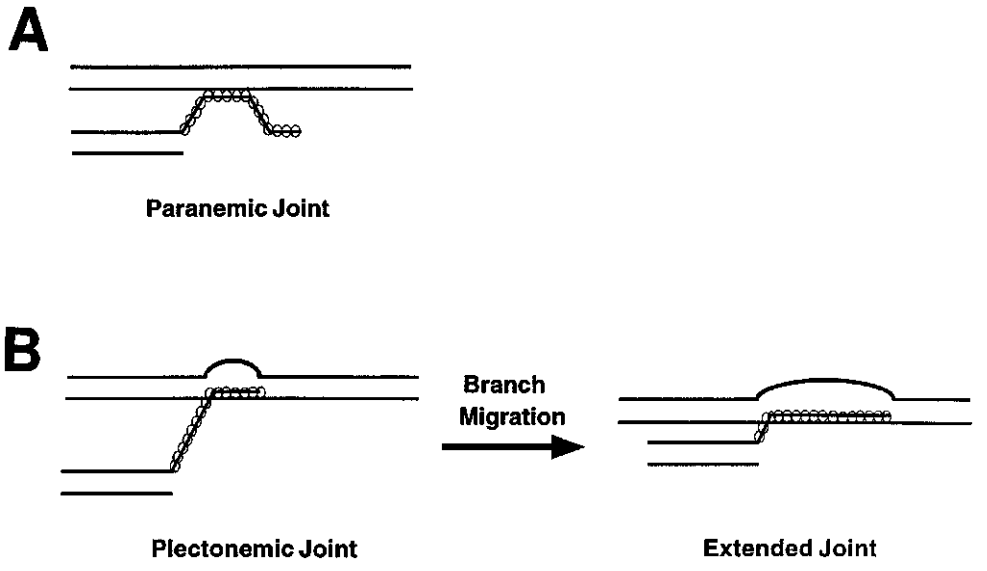

Fig. 3. DNA joints in the formation of heteroduplex DNA. **(A)** The first homologous joints formed between the recombining DNA molecules are paranemic in nature. The paranemic joints (only one such joint is shown) are unstable but are thought to be important intermediates that lead to the capture of the incoming homolog and the homologous alignment of the recombining DNA molecules. **(B)** Subsequent to homologous alignment of the DNA molecules, the free DNA end is located and the formation of a stable plectonemic linkage becomes possible. Branch migration results in extension of the plectonemic joint and the heteroduplex DNA region. The circles in (A) and (B) denote Rad51 molecules. It should be emphasized that the nucleoprotein complex that conducts the DNA homology search and pairing steps very likely contains other protein factors of the *RAD52* group, including Rad52, Rad54, and Rad55-Rad57 in yeast (*see* text for details).

depression of homologous DNA pairing and strand exchange by RPA suggests that specific ancillary factors must function with Rad51 in vivo to overcome the competition posed by RPA. Indeed, the Rad52 protein and the Rad55/Rad57 complex have been shown to facilitate the assembly of the Rad51-ssDNA nucleoprotein filament when RPA is competing for DNA binding sites with Rad51 *(74,100,109,110).* Consistent with the biochemical results, formation of meiotic Rad51 nuclear foci, believed to be sites of ongoing recombination, is strongly dependent on the *RAD52, RAD55,* and *RAD57* genes *(36).* The relevant information concerning these mediators is described in Subheading 3.2.1.–3.2.2.

3.2.1. Rad52 Protein

Rad52 is a ssDNA binding protein *(73,101)* and it forms a complex with Rad51 *(69,98,110).* Addition of Rad52 to a homologous DNA pairing and strand-exchange reaction overcomes the inhibition posed by RPA *(74,100,110),* indicating a mediator function in the protein. Rad52 is of much lower cellular abundance than Rad51, and interestingly, an amount of Rad52 approx one tenth that of Rad51 is optimal for its mediator function *(110).* In the presence of Rad52, RPA is still required for the optimal pairing and strand exchange, thereby excluding the possibility that Rad52 is simply replacing RPA in vitro

(110). Taken together, the results suggest that a complex of Rad51/Rad52 bound to ssDNA provides a priming effect for the recruitment of free Rad51 molecules. Genetic studies have indicated that Rad52 also functions in a specialized pathway of homologous recombination termed single-strand annealing or SSA, which involves the annealing of homologous DNA single strands (*45,77,* and *see* Subheading 6.). Consistent with the genetic results, Rad52 mediates the annealing of DNA strands *(73),* in a reaction that is stimulated by RPA *(101,104).* Recent studies have shown that human Rad52 binds dsDNA breaks and promotes end-to-end DNA interactions in vitro *(29).* A model was proposed that Rad52 binds to DSBs in vivo and mediates repair either by SSA mechanism or by Rad51-mediated pathway by recruiting Rad51 to the breaks *(29).* The strand-annealing activity of Rad52 could also be utilized for the formation of a short heteroduplex joint for priming DNA synthesis, which may be important for a mechanism of DNA repair termed DNA break-induced replication or BIR *(65).*

3.2.2. Rad55/Rad57 Complex

Both Rad55 and Rad57 share some limited homology to Rad51, especially within the sequence motifs involved in the binding and hydrolysis of nucleoside triphosphates *(61).* In agreement with two-hybrid results, which suggested an interaction between Rad55 and Rad57 *(41,48),* stoichiometric amounts of these proteins co-immunoprecipitate from yeast cell lysate, indicating that the proteins are stably associated in a complex *(109).* Purification of the Rad55/Rad57 complex has revealed that it is heterodimeric *(109).* The Rad55/Rad57 heterodimer is also capable of overcoming the inhibition by RPA, indicative of a mediator function of the heterodimer in the assembly of the presynaptic Rad51-ssDNA nucleoprotein filament. The Rad55/Rad57 heterodimer is of much lower cellular abundance than Rad51, and amounts of purified Rad55/Rad57 substoichiometric to that of Rad51 are sufficient for mediator function. In the presence of the Rad55/Rad57 heterodimer, RPA is still needed for efficient pairing and strand exchange *(109).* Whether Rad55/Rad57 acts by a mechanism similar to that of Rad52 or by a distinct mechanism remains to be determined. *rad55* and *rad57* single mutants are as defective in recombination and repair as the double mutant, which emphasizes that Rad55 and Rad57 function together in the same step during recombination, and also nicely underscores the finding that the two proteins are associated as a heterodimer. A notable feature about the *rad55* and *rad57* mutants is that they are cold sensitive for recombination *(62).*

3.3. Factors That Function in the Synaptic Phase

3.3.1. Rad54 Protein

Rad54 belongs to the Swi2/Snf2 protein family, members of which are involved in diverse chromosomal processes including transcription and repair *(30,80,94).* Rad54 is of much lower cellular abundance than Rad51 *(47).* Consistent with the presence of Walker-type nucleotide binding motifs in Rad54 *(31),* purified Rad54 has a robust ATPase activity that is completely dependent on DNA, dsDNA in particular, for its activation. However, Rad54 does not appear to possess a classical DNA helicase activity *(83).* Rad54 physically interacts with Rad51 *(20,47,83),* and the addition of Rad54 protein to a homologous DNA pairing reaction results in a dramatic stimulation of the pairing rate *(83).* Whereas Rad51 is incapable of mediating pairing between a linear ssDNA

molecule and a covalently closed duplex to form a D-loop, the inclusion of Rad54 protein renders D-loop formation highly efficient. Control experiments have shown that Rad54 by itself is devoid of homologous DNA pairing activity *(83).* Human Rad54 also interacts with hRad51 *(38),* possesses a dsDNA-dependent ATPase activity *(111),* and its expression in a yeast *rad54* mutant partially complements the MMS sensitivity of the mutant *(52).* Mouse Rad54 forms nuclear foci that co-localize with mouse Rad51 upon treatment of cells with ionizing radiation, and the formation of the DNA damage-induced Rad51 nuclear foci is dependent on Rad54 *(114).* Human Rad54 induces a conformational change in the duplex DNA in an ATP-hydrolysis-dependent manner, as revealed in a topological unwinding assay *(114).* Yeast Rad54 has also been found to induce a similar conformational change in duplex DNA upon hydrolyzing ATP *(84).* The ability to alter the conformation of the DNA double home is likely to be important for the promotion of the homologous DNA pairing reaction *(84).*

3.1.2. Rdh54 Protein

The yeast Rdh54/Tid1 shows 35% identity to Rad54. Although the *rdh54*Δ mutation confers only slight sensitivity to MMS, it increases the MMS sensitivity of a *rad54*Δ strain. Likewise, the *rad54*Δ *rdh54*Δ double mutant is more impaired in meiosis than either single mutant alone *(55,99).* In a separate study, Dresser et al. *(28)* identified Rdh54 in a two-hybrid screen as a protein that interacts with the meiosis-specific Rad51 homolog Dmc1. These observations suggest that Rdh54 functions with Rad51 in gene conversion between homologs in mitotic cells *(55)* and with both Dmc1 and Rad51 in interhomolog recombination in meiotic cells *(55,99).* Like Rad54, Rdh54 has Walker-type nucleotide binding motifs. Considering the involvement of Rdh54 in recombination and repair as well as its structural similarity to Rad54, one suspects that Rdh54 also affects heteroduplex DNA formation during the synaptic phase.

3.2. Other Recombination Factors

3.2.1. Rad59 Protein

Rad59 functions in intrachromosomal recombination and DNA DSB repair, apparently in a pathway distinct from that dependent on Rad51. Rad59 shows some homology to Rad52, and overexpression of *RAD52* partially suppresses the recombination deficiency and γ-ray sensitivity of a *rad59* mutant *(4).* The biochemical function of Rad59 protein in recombination and repair remains to be determined.

3.2.2. Dmc1, a Meiosis Specific Recombinase

Yeast Dmc1 is highly homologous to Rad51. *DMC1* is required for meiotic recombination and chromosomal disjunction, and its expression is restricted to meiosis. Consistent with this expression pattern, deletion of *DMC1* produces no discernible mitotic phenotype *(11).* Dmc1 colocalizes with Rad51 in nuclear foci during meiosis *(10).* Dmc1 structure and function appear to be highly conserved among eukaryotic organisms *(85,124).* Human Dmc1 has been purified and shown to possess homologous DNA pairing activity *(59).* The fact that amounts of hDmc1 stoichiometric to that of ssDNA are required for the optimal rate of homologous pairing argues for the possibility that hDmc1 promotes pairing via the formation of a stoichiometric protein complex on ssDNA, likely a nucleoprotein filament *(59).* The level of homologous pairing that can be achieved by hDmc1 protein is relatively low, suggesting that ancillary factors may

function with hDmc1 to attain optimal activity. Whether Dmc1 from yeast and human cooperates with the same set of recombination factors that function to enhance the recombinase activity of Rad51 remains to be determined.

3.3 Human Proteins Homologous to Rad51

The chinese hamster ovary (CHO) cell lines irs1 and irs1SF, defective in the *XRCC2* and *XRCC3* genes, respectively, are sensitive to ionizing radiation and to DNA crosslinking agents *(50,115,117).* Analyses of the cloned *XRCC2* and *XRCC3* cDNAs have revealed that their products are homologous to Rad51 *(18,60).* In addition, XRCC3 has been shown to interact with Rad51 in the yeast two-hybrid system and to co-immunoprecipitate with Rad51 from cell extract *(60).* Interestingly, XRCC3 has been shown in the Bishop laboratory to be required for the formation of DNA damage-induced Rad51 nuclear foci *(12).* Taken together, it seems plausible to suggest that XRCC3 functions with Rad51 in recombination and DNA repair processes. Given the phenotypic similarity of the irs1 cell line to the irs1SF cell line and the homology of XRCC2 to XRCC3 and Rad51, it is likely that XRCC2 is also a component of the recombination protein machine that contains the latter two proteins. Three additional Rad51-homologous proteins, Rec2/Rad51B *(3,90),* Rad51C *(27),* and Rad51D *(86),* have been identified based on their amino acid sequence homology to Rad51. Rad51C has been found in the yeast two-hybrid system to interact with XRCC3 and Rad51B *(27).* In *S. cerevisiae,* the Rad51-homologous proteins Rad55 and Rad57 combine to form a heterodimer (*see* Subheading 3.2.2.) that functions to promote the assembly of the Rad51-ssDNA nucleprotein filament *(109).* There is a distinct possibility Rad51B, Rad51C, Rad51D, XRCC2, and XRCC3 also form complexes with one another and function to enhance hRad51-ssDNA nucleoprotein filament assembly.

4. REPAIR DNA SYNTHESIS, RESOLUTION OF RECOMBINATION INTERMEDIATES, AND DNA LIGATION

As can be seen in the model in Fig. 1, concomitant with heteroduplex DNA formation, repair DNA synthesis replaces the genetic information eliminated during the end-processing reaction. Holmes and Haber *(43)* have presented evidence that during conversion of the mating-type information at *MAT,* the repair DNA synthesis step requires the concerted action of DNA polymerases α, δ, and ε, leading to the suggestion that the repair synthesis reaction entails the establishment of both leading and lagging DNA strands. Logic would predicate that the repair DNA synthesis reaction be coupled to the strand-invasion reaction that yields heteroduplex DNA. In other words, it is very likely that the DNA polymerases and accessory factors are actively recruited to the sites of recombination. At present, little is known about how the DNA synthesis proteins are recruited to recombination sites, and if any of the known recombination factors serves to recruit them.

Two genes, *MSH4* and *MSH5,* appear to specifically affect crossover or reciprocal recombination and may therefore encode factors that influence processing of a DNA intermediate, such as the Holliday junction, critical for the generation of crossover recombinants. Expression of *MSH4* and *MSH5* is seen only in meiosis, and in *S. cerevisiae* strains mutated for these two genes, the levels of meiotic-gene conversion and postmeiotic segregation appear to be normal at the majority of the loci examined, but crossover recombination is reduced two- to threefold. As a result of the deficiency in

crossover recombination, *msh4* and *msh5* mutants are partially defective in chromosome disjunction during meiosis I, giving rise to a sporulation deficit and low spore viability. The *MSH4* and *MSH5* genes show epistasis in meiotic crossover recombination, suggesting that their encoded products function in the same biological pathway or reaction *(42,92).* More recent results have indicated that Msh4 and Msh5 exist as a complex in yeast cells *(87)* and have implicated a role for the mismatch repair factor Mlh1 in the Msh4/Msh5-dependent pathway of crossover recombination *(44).* Aside from a possible function of the Msh4-Msh5 protein complex in Holliday-junction processing, relatively little is known about other nuclear recombination factors that promote branch migration and resolution of the Holliday junction and other recombination intermediates.

Following the resolution of recombination intermediates, DNA ligation complete the recombination process by sealing the DNA nicks. Of the two DNA ligases in yeast, ligase I and ligase IV, existing evidence suggests that ligase I, the product of the *CDC9* gene, is involved in recombinational DNA repair *(33).*

5. MODULATION OF DSB REPAIR BY TUMOR SUPPRESSORS

There is emerging evidence that the recombination machinery in mammalian cells is subject to modulation by genes that are critical for cancer suppression. In addition to the *NBS1* gene, which encodes an integral component of the Mre11-associated nuclease complex, recent studies have implicated the gene mutated in ataxia-telangiectasia *(ATM)* and the breast tumor-suppressor gene *BRCA2* in recombination and repair.

Ataxia-telangiectasia (A-T) is an autosomal recessive disease characterized by neurodegeneration, cancer predisposition, immunodeficiency, gonadal atrophy, and hypersensitivity to ionizing radiation (reviewed in *58* and *68). ATM* encodes a member of the PI3-like kinase family that includes proteins involved in cell-cycle checkpoint control, meiosis, and V(D)J recombination (reviewed in *125*). ATM protein phosphorylates p53, and this modification is believed to be important for the p53-dependent DNA damage induced G1/S checkpoint *(5,15).* However, because p53 is not essential for other ATM-mediated checkpoints, it is likely that other checkpoint factors are also modified in an ATM-dependent manner. In addition to its checkpoint function, ATM may be directly involved in DNA repair processes, as A-T cells remain hypersensitive to ionizing radiation under conditions where the checkpoint function is dispensable *(116).* Furthermore, most A-T patients and all ATM-deficient mice of both sexes are infertile owing to the absence of mature gametes (reviewed in *58).* In mice, ATM is required for meiosis as early as the leptonema stage of meiosis I. In the absence of ATM, Dmc1 and Rad51 mislocalize to chromatin instead of the developing synaptonemal complex *(6).* The mislocalization of Rad51 appears to reflect a requirement for the ATM-dependent kinase cascade in the phosphorylation of Rad51 protein (EL, unpublished observation). A high frequency of spontaneous recombination has been observed in A-T cells *(63,67),* and in addition, DNA end-joining also appears to be aberrant in these cells *(24).* It is interesting to note that A-T and NBS cells share many cellular phenotypes, thus NBS was long-regarded as an A-T variant (reviewed in *97).*

Mutations in *BRCA2* account for a large proportion of familial breast cancers (reviewed in *126*). Cultured cells become sensitive to γ-irradiation upon downregulation of BRCA2 protein expression by treatment with BRCA2 antisense oligonucleotides *(1).* In addition, fibroblasts established from mutant *Brca2* mouse embryos are specifically

sensitive to γ-irradiation *(22,72,76).* BRCA2 protein associates with Rad51, and the interaction domain has been mapped to the BRC repeats in BRCA2 *(19,53,122).* The human pancreatic adenocarcinoma Capan-1 cells, which lack one copy of the *BRCA2* gene and contain a truncating mutation (6174delT) in the other *BRCA2* allele *(37),* are hypersensitive to various DNA damaging agents. The MMS sensitivity of Capan-1 cells is complemented by the introduction of *BRCA2* cDNA, but not by a truncated cDNA with the BRC repeats deleted. These observations have provided evidence that the interaction between Rad51 and the BRC repeats in BRCA2 is important for DNA repair *(19).*

6. HOMOLOGOUS RECOMBINATION BY SINGLE-STRAND DNA ANNEALING

When homologous genetic elements are arranged as direct repeats on the same chromosome or DNA molecule, a plasmid for instance, a DSB introduced between the DNA repeats is processed to yield 3′ overhanging single-stranded tails that are partially complementary to each other, and thus have the potential of undergoing annealing to yield heteroduplex DNA. Once hybridized, the remaining nonhomologous, overhanging single-stranded tails are trimmed nucleolytically. Gap filling by a DNA polymerase followed by DNA ligation would yield a recombinant DNA molecule that has some of the original DNA sequence deleted. This type of homologous recombination is termed single-strand annealing or SSA *(45,77).* Interestingly, the SSA pathway of recombination shows a dependence on *RAD52* but not on the other members of the *RAD52* epistasis group, with the possible exception of *RAD59.*

7. EPILOGUE

Now that many of the main players that stage the production of DNA DSB, DNA end-processing, and heteroduplex DNA formation have been identified in *S. cerevisiae,* it is expected that rapid progress will be made on understanding these individual enzymatic steps at the mechanistic level. Major challenges lie with identifying the protein components involved in the later stages of recombination processes. Attempting to elucidate the mechanism of action of various tumor suppressors in modulating the efficiency of the DNA DSB repair machinery will be a highly interesting area of research in the coming years.

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