Investigation of the Stability of Yeast rad52 Mutant Proteins Uncovers Post-translational and Transcriptional Regulation of Rad52p

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ABSTRACT

We investigated the stability of the Saccharomyces cerevisiae Rad52 protein to learn how a cell controls its quantity and longevity. We measured the cellular levels of wild-type and mutant forms of Rad52p when expressed from the RAD52 promoter and the half-lives of the various forms of Rad52p when expressed from the GAL1 promoter. The wild-type protein has a half-life of 15 min. rad52 mutations variably affect the cellular levels of the protein products, and these levels correlate with the measured half-lives. While missense mutations in the N terminus of the protein drastically reduce the cellular levels of the mutant proteins, two mutations—one a deletion of amino acids 210–327 and the other a missense mutation of residue 235—increase the cellular level and half-life more than twofold. These results suggest that Rad52p is subject to post-translational regulation. Proteasomal mutations have no effect on Rad52p half-life but increase the amount of RAD52 message. In contrast to Rad52p, the half-life of Rad51p is >2 hr, and RAD51 expression is unaffected by proteasomal mutations. These differences between Rad52p and Rad51p suggest differential regulation of two proteins that interact in recombinational repair.

The RAD52 epistasis group is required for DNA double-strand-break repair and for recombination in yeast. This group of genes currently consists of RAD50–57, RAD59, RAD54, RFA1, MRE11, and XRS2. Together these genes are thought to act sequentially or as part of a larger recombinational repair complex to promote double-strand break repair. A direct physical or a genetic interaction between a number of these genes has been demonstrated, suggesting that a multiprotein complex is required for efficient recombinational repair (Milne and Weaver 1993; Firmenich et al. 1995; Johnson and Symington 1995; Schiødt 1995; Bai and Symington 1996; Hays et al. 1998; Shinohara and Ogawa 1998). This multiprotein complex is likely a dynamic structure that is remodeled and subjected to a series of rearrangements during the process of double-strand-break repair. How this complex forms, changes, and disassembles is not currently well understood.

While deletions of any of the genes in the RAD52 epistasis group cause a wide array of deficiencies associated with the inability to repair a DNA double-strand break, deletion of RAD52 produces the most severe phenotypic defects. These include extreme sensitivity to ionizing radiation or radiomimetic chemicals, a reduced level of mitotic recombination, and an inability to undergo sporulation. Unlike other members of the RAD52 epistasis group, RAD52 is required for repair not only by homologous recombination but also by single-strand annealing (Ivanov et al. 1996), chromosomal inverted repeat recombination (Rattray and Symington 1994), and break-induced replication (Malkova et al. 1996; Signon et al. 2001), processes that do not rely on RAD51, RAD53, or RAD57. Thus, genetic analysis indicates that Rad52p has a central role in many forms of recombinational repair.

In vitro studies using the purified Rad52 protein have identified at least two independent functions of Rad52p that make it absolutely essential for recombinational repair. First, the amino terminus of Rad52p is capable of binding single- and double-strand DNA and of promoting DNA strand annealing between two homologous DNA molecules (Mortensen et al. 1996). This activity of Rad52p explains why RAD52 is required not only for homologous recombination, but also for other repair processes such as single-strand annealing and break-induced replication. Second, Rad52p is required to serve as a mediator between RPA (replication protein A) and Rad51p at the double-strand-break site (Song and Sung 2000). Although RPA and Rad51p compete for binding to the single-strand DNA (ssDNA) tails at the break site, in vitro strand exchange occurs most efficiently in the presence of both RPA and Rad51p. This reaction is completely dependent on Rad52p, suggesting that it functions to mediate the interaction between RPA and Rad51p (Song and Sung 2000). In fact, Rad52p physically interacts with all three subunits of RPA (Hays et al. 1998), as well as with Rad51p (Milne and Weaver 1993; Donovan et al. 1994; Hays et al. 1995). The results of the strand exchange experiments and the physical interactions that have been identified...
TABLE 1

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MATERIALS AND METHODS

Yeast strains: All yeast strains used in this study are isogenic with SSI204 (DORNFIELD and LIVINGSTON 1991) unless otherwise noted and are listed in Table 1. The rad52 mutant alleles employed in this study are shown in Figure 1.

Plasmids: To place an epitope tag at the amino terminus of Rad52p, RAD51 was mutagenized to introduce a Nol restriction site immediately after the start codon. The sequence bearing three copies of the flag peptide was inserted in frame into this site. The flag-tagged version of RAD51 was placed into pRS814 (SIKORSKI and HEITER 1989) using the BamHI and Pdr sites. This construct was found to confer increased methyl methanesulfonate (MMS) resistance to a rad51Δ strain. From this construct, RAD51::FLAG3 was amplified by PCR using primers that introduced a BamHI site flanking the RAD51 coding sequence at both the 5’ and 3’ ends. This PCR product was digested with BamHI and cloned into the CEN-based plasmid pBM272 (Mark Johnston, Washington University, St. Louis). pBM272 is a derivative of pB150 (JOHNSTON and DAVIS 1984) and has a BamHI site downstream of the yeast GAL1 promoter. To place an epitope tag at the carboxy terminus of Rad52p, the stop codon and adjacent sequence were mutagenized to include a Nol restriction site at the end of the coding sequence. The sequence bearing three copies of the flag peptide was inserted into this Nol site. The extended RAD52 gene with the epitope tag was found to confer MMS resistance to a strain deleted for RAD52, indicating that the tag does not interfere with RAD52 activity. A fragment of RAD52 from this clone was subcloned into pDML5 (DORNFIELD and LIVINGSTON 1991) using restriction endonuclease digestion by BstEII and SalI. This placed the flag-tagged version of RAD52 downstream of the yeast GAL1 promoter at the HindIII site of pBM272. The C-terminal truncation and internal deletion alleles and several missense mutations were subcloned into this construct using restriction endonuclease digestion by BstEII and SphI of the pRS166 constructs containing these rad52 mutations (ASLESON et al. 1999), GAL1-promoted, flag-tagged RAD52 was also subcloned into pRS314 (SIKORSKI and HEITER 1989) from pBM272 by restriction endonuclease digestion with EcoRI and SalI.

UBC9was cloned by PCR using primers that introduce EcoRI sites at the 5’ and 3’ ends of the gene. This PCR product was digested with EcoRI and cloned into pRS424 (CHRISTIANSON et al. 1992), which was linearized with the same restriction endonuclease. The UBC9 clone was confirmed by sequencing. The RAD51 and RFA2 genes were cloned by PCR using primers that introduce BamHI sites flanking the 5’ and 3’ ends of the genes. Following digestion with BamHI, both RAD51 and RFA2 were cloned into the BamHI site of pRS425 (CHRISTIANSON et al. 1992). The RAD51 clone was tested by confirming its ability to confer MMS resistance to a rad51Δ strain. The RFA2 clone was confirmed by complementation of a rfa2-2 strain at 37°C (SANTOCANAŁE et al. 1995). For the studies using overexpression of RAD51, UBC9, or RFA2, rad52ΔHS was transformed with the CEN-based plasmid pRS316 carrying the rad52
Regulation of Rad52p

Figure 1.—rad52 alleles used in this study. The relative positions of the missense mutations are indicated above the RAD52 opening reading frame represented by the arrow. The internal deletion mutations are represented below. The cross-hatched region represents the relative position of the in-frame deletion in each of the mutant proteins. The mutational changes present in each of these alleles are listed below the diagram. rad52-301A is a cold-sensitive missense mutation (Nguyen and Livingston 1997b), rad52-2 was previously described (Strike 1978; Adzuma et al. 1984; Boundy-Mills and Livingston 1993), and rad52-763a and rad52-96a are temperature-sensitive mutations in RAD52 (Kaytor and Livingston 1994; Asleson et al. 1999). rad52-Δ210B and rad52-Δ292B are internal deletion alleles previously described (Asleson et al. 1999), and the two other internal deletion alleles, rad52-Δ210-251 and rad52-Δ251-292, were constructed for this study.

Allele and either pRS424 carrying UBC9 or pRS425 carrying RFA2 or RAD51. These strains were grown on complete agar lacking uracil and tryptophan or uracil and leucine, respectively, and in SC-Ura-Trp or in SC-Ura-Leu liquid media.

Growth conditions for determination of mRNA and protein cellular levels: To determine chromosomal mRNA cellular levels, cells were grown in YM-1 liquid media. To determine protein cellular levels, the CEN-based plasmid pRS316 (Sikorski and Hieter 1989) carrying flag-tagged RAD52 or rad52 mutant alleles under the control of their own promoter were transformed into SSLA35 (pup1-1 rad52ΔHIS) and SSL212 (rad52ΔHIS), along with a second CEN-based plasmid carrying a flag-tagged copy of the splicing factor, EXO84, to serve as a loading control for Western blotting (Guo et al. 1999; Awasthi et al. 2001). These strains were grown on complete agar plates lacking uracil and histidine or in SC-Ura-His liquid media to maintain the plasmids.

Growth conditions for determination of mRNA and protein half-lives: The plasmid containing GAL1-promoted RAD52 or one of the rad52 mutant alleles was transformed into SSLI212 (rad52ΔHIS) and the plasmid containing GAL1-promoted RAD51 was transformed into SSL286 (rad51:ADE2). A second CEN-based plasmid, carrying a flag-tagged copy of the splicing factor, EXO84, under its own promoter was transformed along with the GAL1-promoted constructs to serve as a detectable control for equal loading in Western blotting. For the individual determination of either Rad51p or Rad52p half-life, each strain was grown at 30° to early log phase in minimal media lacking uracil and histidine buffered to a pH of 4.0 with succinic acid and containing 2% raffinose as a carbon source. To induce expression from the GAL1 promoter, galactose was added to 2% and the culture was allowed to grow at 30° to 1–3 × 108 cells/ml, or ~4 hr. To repress the galactose induction, and thereby shut off RAD51 or RAD52 production, the cells were collected by centrifugation and resuspended in SC-Ura-His liquid media containing 2% glucose. Samples of cells (2 × 107 cells) were removed at various times, collected by centrifugation, and resuspended in yeast lysis buffer for either protein extraction (Katz and Solomon 1988) or RNA extraction (Brill and Sternglanz 1988) using a glass bead breakage method. Both GAL1-promoted RAD51-FLAG3 carried on pBM272 and GAL1-promoted RAD52-FLAG3 carried on pRS14 were also transformed into SSL725 (rad51::ADE2 rad52ΔHIS). This strain was grown in media lacking uracil and tryptophan and used to determine the half-lives of both RAD51 and RAD52 mRNAs and proteins simultaneously from the same extracts while both proteins were being overexpressed.

Western blot analysis: Equal amounts of protein from each time point, determined by using the Bio-Rad (Hercules, CA) protein concentration assay, were separated by electrophoresis in a SDS gel. The proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH) and flag-tagged polypeptides were detected by incubating the membranes with mouse anti-flag M2 monoclonal antibody (Sigma-Aldrich, St. Louis), followed by incubation with goat anti-mouse IgG conjugated to horseradish peroxidase (HRP; Bio-Rad). Proteins were visualized using HRP substrate reagents followed by exposure to hyperfilm-enhanced chemiluminescence according to the manufacturer’s instructions (Amersham Pharmacia, Buckinghamshire, UK). The resulting bands on hyperfilm were quantitated by using a computing densitometer (Molecular Dynamics, Piscataway, NJ). Except where noted, the results from six to nine experiments were averaged and plotted to determine the relative half-life of each protein.

Northern blot analysis: The concentration of RNA was determined by finding the OD260 using a DU 640 spectrophotometer (Beckman, Fullerton, CA). Equal amounts of total cellular RNA were separated on a 1.5% formaldehyde agarose gels. The gels were washed and the RNA was transferred to nitrocellulose membranes (Schleicher & Schuell) in 20× SSC. The membranes were dried and the RNA was crosslinked to the membrane using a GS genelinker UV chamber (Bio-Rad). The membranes were incubated in prehybridization buffer containing 6× SSC, 5× Denhardt’s reagent, 0.5% SDS, and 100 μg/ml salmon sperm DNA (Maniatis et al. 1989) at 60° for 2 hr. The membranes were probed overnight at 42° in hybridization buffer containing 6× SSC, 50% formamide, 0.5% SDS, and 100 μg/ml salmon sperm DNA (Maniatis et al. 1989). The membranes were washed at room temperature twice for 30 min each in 2× SSC and 0.1% SDS and once at 65° for 45 min in 0.1× SSC and 0.1% SDS. RNA was visualized and quantitated using a Phosphorimager 445 SSI system and IP lab gel software (Signal Analytics, Vienna, VA). The probe used to detect RAD52 mRNA was a HpaII-SphI fragment. The probe used to detect RAD51 mRNA was a 2.1-kb BamHI-HindIII fragment that included the entire RAD51 coding sequence. A probe to ACT1 was generated by PCR to serve as a control for mRNA expression. The results of three experiments were averaged to determine the relative half-lives of each mRNA.

RESULTS

Mutant forms of Rad52p have variable cellular levels: We previously observed that mutations in RAD52 variably affect the cellular level of the protein product, Rad52p (Asleson et al. 1999). Because in our previous report we had expressed RAD52 (or mutated copies)
from high-copy plasmids, we repeated the analysis by examining the expression of specific alleles from single-copy plasmids. We found that the variability in quantity is the same whether expression is from high-copy or single-copy plasmids. While some missense mutations in the amino terminus of Rad52p, rad52-2 (P64L) and rad52-76A (N97T), decrease the cellular level of the protein, a deletion mutation that removes the entire internal region of Rad52p from amino acids 210–327, rad52-d210B, increases the cellular level of the protein more than twofold (Figure 2). rad52-96A (R235G), a missense mutation located in this same internal region of Rad52p, also leads to the production of a mutant protein that is more abundant than the wild-type Rad52 protein (Figure 2).

These observations show that the quantities of wild-type and mutant Rad52 proteins are consistent relative to each other whether the proteins are being overexpressed from a high-copy plasmid or expressed at normal cellular levels from a single-copy plasmid. Furthermore, the fact that mutations in this middle region of Rad52p stabilize the protein, while others located in the amino terminus either destabilize the protein or have no effect on its cellular level, suggests that this region may play a role in the regulated turnover of Rad52p.

Rad52p has a short half-life relative to Rad51p: To understand the cause in variability of the cellular levels of Rad52 mutant proteins, we examined whether the half-lives of the mutant proteins correlate with their cellular levels. As a starting point, we measured the half-life of wild-type Rad52p relative to the half-life of Rad51p. Flag-tagged versions of RAD52 or RAD51 were cloned behind the inducible GAL1 promoter. The plasmids containing RAD52 or RAD51, along with a second plasmid containing a flag-tagged copy of an unrelated gene that served as a loading control for Western blotting, were transformed into a rad52Δ strain or a rad51Δ strain, respectively. The strains were grown under the appropriate conditions to induce and then to repress expression from the GAL1 promoter. Cells were collected at specific times following repression of the GAL1 promoter, proteins were extracted, and Rad52p or Rad51p was detected by Western blotting with an anti-flag antibody. The half-life of Rad52p is 15 min (Figure 3B). In contrast, the half-life of Rad51p is between 2.5 and 3 hr (Figure 3B). The half-life value for Rad51p may result from dilution during cellular growth and not from degradation. In the case of Rad52p the half-life is considerably less than the doubling time and reflects degradation.

Because protein half-lives are partially dependent on the half-lives of the corresponding mRNAs, we also determined the half-lives of RAD51 and RAD52 mRNA. The RAD52 mRNA half-life is between 2 and 4 min, showing that the half-life of Rad52p is not limited exclusively by the half-life of its message (our unpublished data). In contrast, the half-life of RAD51 mRNA is between 16 and 20 min (our unpublished data). Thus, while the mRNA half-lives of RAD52 and RAD51 correlate with their protein half-lives, the difference in the half-lives of the two messages does not fully account for the difference in the half-lives of the two proteins. The differences between Rad52p and Rad51p in both their
Regulation of Rad52p

The first deletion extended from amino acid 210 to 251, the second from 251 to 292, and the third from 292 to 327. All three internal deletions within the region from amino acids 210 to 327 of Rad52p extended the half-life of the protein more than twofold (Figure 4A and our unpublished data). To ensure that the increase in the half-lives of these mutant proteins resulted from altered post-translational regulation rather than from mRNA stability, the half-lives of the mRNAs expressed from the GAL1 promoter were compared to the half-life of the wild-type Rad52 mRNA. No significant differences were apparent between the mRNA half-lives of Rad52 and any of the internal deletion alleles, confirming that the effect on protein half-life does not result from an increase in mRNA stability (our unpublished data).

A missense mutation in amino acid 235 extends the half-life of Rad52p: In our earlier work, we isolated and characterized a missense mutation, rad52-96A (R235G), which lies within the middle of Rad52p (Figure 1; Asleson et al. 1999). The rad52-96A mutant is partially sensitive to MMS at 25°C and fully sensitive at 33°C. Its MMS susceptibility is suppressible by high-level expression of RAD51. Because of the location of the mutation and the elevated cellular level of the protein, we measured the protein’s half-life. Strikingly, the half-life of Rad52-96Ap is similar to that of the mutant proteins containing internal deletions between amino acids 210 and 327 and is increased more than twofold over the half-life of wild-type Rad52p (Figure 4B). This result is novel because other full-length, missense mutant proteins either have a half-life that is slightly shorter than that of wild-type Rad52p, e.g., Rad52-76Ap (N97T; Figure 4B), or are so unstable that they cannot be detected even when expressed from the GAL1 promoter, e.g., Rad52-2p (P64L; our unpublished data).

The relative half-lives of the wild-type and mutant Rad52 proteins correlate with the differences in their cellular quantities (Figure 2). Mutants that are present in greater quantity also have longer half-lives. This correlation indicates that the cellular quantities are, at least in part, a function of the intrinsic half-lives of the proteins.

High-level expression of Rad52p does not alter the cellular amount of wild-type or mutant proteins: To directly address the issue of the effect of high-level expression on protein longevity, we examined the cellular quantity of epitope-tagged Rad52 proteins in cells expressing high levels of untagged wild-type protein. The results (Figure 5) show that high-level expression of the untagged protein has no effect on the cellular quantity of the epitope-tagged proteins expressed in normal cellular amounts from the RAD52 promoter on a single-copy plasmid. Considering that epitope-tagged Rad52p is fully functional and, consequently, likely to mix freely with the pool of untagged proteins, the results indicate that high-level expression used in the half-life studies does not alter the intrinsic stability of the Rad52 pro-

mRNA and protein half-lives indicate that the two proteins are not subject to identical regulation.

Internal deletions in RAD52 extend the half-life of the protein: We next measured the half-lives of mutant forms of Rad52p. We began with the protein product of rad52-210B, which has a cellular level more than twice as high as that of the wild-type protein. This internal deletion protein has a half-life more than twofold longer than that of wild-type Rad52p (Figure 4A).

To map this effect in the middle section of Rad52p, three smaller deletions of 40 amino acids were constructed. The first deletion extended from amino acid 210 to 251, the second from 251 to 292, and the third from 292 to 327. All three internal deletions within the region from amino acids 210 to 327 of Rad52p extended the half-life of the protein more than twofold (Figure 4A and our unpublished data). To ensure that the increase in the half-lives of these mutant proteins resulted from altered post-translational regulation rather than from mRNA stability, the half-lives of the mRNAs expressed from the GAL1 promoter were compared to the half-life of the wild-type Rad52 mRNA. No significant differences were apparent between the mRNA half-lives of Rad52 and any of the internal deletion alleles, confirming that the effect on protein half-life does not result from an increase in mRNA stability (our unpublished data).

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teins. If Rad52p in excess of normal cellular amounts had either a shorter or a longer half-life than that of Rad52p in its normal cellular amount, then the cellular level of the epitope-tagged protein expressed at normal levels and mixed with an excess of untagged protein would have been in either lesser or greater quantity, respectively.

Factors that do not affect the stability of Rad52p: In an attempt to identify proteins that interact with Rad52p and that control its degradation, we concentrated our efforts on obvious candidates: either proteins known to interact with Rad52p or proteins known to play a role in protein degradation. We tested the involvement of other proteins in Rad52p degradation either by measuring the half-life of Rad52p in the presence of mutations in a second gene or by analyzing the cellular levels of Rad52p or Rad52-96Ap in the presence of high-copy expression of a second gene (Table 2). We hypothesized that loss-of-function mutations in a gene that regulates Rad52p turnover will increase its half-life, while overexpression of this gene may decrease the cellular levels and the half-lives of both Rad52p and Rad52-96Ap.

Because Rad52p physically interacts with Rad51p (Milne and Weaver 1993; Donovan et al. 1994; Hays et al. 1995), we analyzed the cellular level of wild-type Rad52p and several mutant forms with and without high-copy expression of RAD51. High-copy expression of RAD51 from a 2 μ-based plasmid had no effect on the levels of wild-type or mutant proteins (Figure 6A). To extend this result, we also measured the half-life of Rad52p in rad51Δ cells and in cells where RAD51 was overexpressed from the GAL1 promoter (Figure 3A). Simultaneous overexpression of both proteins was important in case the two proteins need to be stoichiometrically equivalent for proper degradation to occur. Again, there was no change in Rad52p half-life in either of these experiments, consistent with the measurement of its cellular level (our unpublished data).

RPA also physically interacts with Rad52p (Hays et al. 1998). To determine whether RPA affects Rad52p stability, we measured the half-life of Rad52p in strains carrying rfa1 and rfa2 mutations. rfa1-t33 is a temperature-sensitive mutation located in the largest subunit of RPA (Umezu et al. 1998). rfa2-1 and rfa2-2 are both temperature-sensitive mutations in the middle subunit of RPA (Santocanale et al. 1995). The rfa2-2 mutation introduces a premature stop codon that creates a mutant protein missing the C-terminal 101 amino acids of Rfa2p. More importantly, this mutation leads to unstable Rfa2p, suggesting that very little heterotrimeric RPA forms in this mutant. None of these rfa mutations had an effect on Rad52p half-life (our unpublished data) when grown at the semipermissive temperature. These experiments were complicated by the fact that the rfa1 and rfa2 mutations make cells grow slowly and necessitate an increase in the time of galactose induction. For this reason, we also tested the effect of excess RFA2 on the cellular level of Rad52p. High-copy expression of RFA2 from a 2 μ-based plasmid again had no effect on

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cally interact with Rad52p in yeast or mammals alters the stability of Rad52p.

A mutation in the proteasome increases the cellular levels of Rad52p and all mutant forms of Rad52p not by stabilizing the proteins but by increasing RAD52 mRNA levels: One mutation that was found to increase the cellular level of Rad52p is in the proteasome, pup1-1. PUP1 is a gene encoding a protease subunit of the 20S proteasome (Hilt et al. 1993), and pup1-1 was isolated and cloned as an allele-specific suppressor of the temperature-sensitive missense mutation rad52-76A (Nguyen and Livingston 1997). We hypothesized that the pup1-1 mutation acts as a suppressor by stabilizing fragile mutant forms of Rad52p. We first determined the cellular levels of Rad52p and several mutant forms of Rad52p in pup1-1 cells. The pup1-1 mutation increases the cellular levels of wild-type Rad52p and all mutant forms of Rad52p greater than twofold over the levels detected in the PUP1 background (Figure 7A). We went on to determine the half-life of wild-type Rad52p in the pup1-1 mutant to confirm that the proteasome was acting directly on Rad52p, but surprisingly, the half-life of Rad52p is not increased in this background (Figure 7B).

Because the half-life of Rad52p does not increase in the pup1-1 mutant, we determined whether RAD52 mRNA levels are increased in pup1-1 cells. In the pup1-1 mutant RAD52 mRNA levels are increased twofold relative to mRNA levels in the control strain. In contrast, neither the quantity of RAD51 nor the level of ACT1 mRNA is increased in the pup1-1 mutant (Figure 8A). To further ensure that this effect is specific to RAD52, we analyzed the mRNA levels of RAD59, a RAD52 paralog; CDC9, encoding DNA ligase I; and PRI2, encoding a primase subunit. The pup1-1 mutation does not affect the mRNA levels of any of these genes (our unpublished data). To further confirm that the proteasome specifically affects the quantity of RAD52 mRNA, we analyzed RAD52 mRNA levels in two other proteasomal mutants, cin5-1 and pre1-1 pre2-2 (Ghislain et al. 1993; Heinemeyer et al. 1993). In strains carrying these mutations, as in the pup1-1 mutant, RAD52 mRNA levels are increased twofold, while RAD51 and ACT1 mRNA levels are unaffected (Figure 8B).

These results suggest that the proteasome affects Rad52 protein levels by acting on a factor involved in RAD52 transcription or in RAD52 mRNA stability. To distinguish between these two possibilities, we compared the half-life of RAD52 mRNA in a wild-type and in a pup1-1 strain. pup1-1 does not elevate the half-life of the RAD52 mRNA, demonstrating that this proteasomal mutation does not affect RAD52 mRNA stability (our unpublished data). Therefore, this result suggests that the proteasome acts on a factor involved in the regulation of RAD52 transcription initiation. A likely scenario is that RAD52 mRNA levels are increased in the presence of proteasomal mutations because, under these conditions, this normally labile transcription factor is stabilized.

the stability of Rad52p (Figure 6C). Thus, RPA does not regulate the stability of Rad52p.

Rad52p also interacts with its paralog Rad59p (Bai and Symington 1996; Davis and Symington 2001). In a strain deleted for both RAD52 and RAD59, the half-life of epitope-tagged Rad52p was not altered (our unpublished data), showing that Rad59p does not affect the longevity of Rad52p.

As a test to understand whether DNA damage alters the half-life of Rad52p, we carried out a half-life study in the presence of MMS. The radiomimetic agent did not change Rad52p’s half-life. Thus, damaged DNA that is the site of Rad52p localization (Lisby et al. 2001) does not change the protein’s stability.

Finally, we tested the role of UBC9 in the stability of Rad52p. Ubc9p is the yeast ortholog of mammalian Ube1p, which has been shown to physically interact with the human Rad52p and Rad51p orthologs (Shen et al. 1996a,b). The protein products of UBE12 and UBC9 conjugate the ubiquitin paralog SUMO-1 in mammals and Smt3p in yeast, respectively (Johnson and Blobel 1997; Schwarz et al. 1998). High-copy expression of UBC9 from a 2 μ-based plasmid did not affect the cellular level of wild-type or mutant Rad52 proteins (Figure 6B).

Consequently, none of the proteins known to physi-
Figure 7.—*pup1-1* increases the cellular level of Rad52p and all mutant forms, but does not increase the half-life of Rad52p. (A) Cellular levels of Rad52p and mutant forms of Rad52p in a strain containing a mutation in the proteasome. Flag-tagged Rad52p or one of the mutant forms of Rad52p carried on a *CEN*-based plasmid were transformed into a *pup1-1 rad52Δ* strain along with a control plasmid carrying Exo84p. Proteins were extracted from log-phase cultures growing at 30°C and the amount of protein was compared to the amount extracted from a *PUP1 rad52Δ* strain carrying the same plasmids. (B) Half-life of Rad52p in *pup1-1 rad52Δ*. The half-life of Rad52p was determined in a strain containing a mutation in the proteasome. The results of three independent experiments were averaged and plotted alongside the half-life of Rad52p in a *PUP1 rad52Δ* strain.

**DISCUSSION**

With this study we initiated an investigation of how the level of Rad52p is regulated by the cell. We made the observation that the cellular levels of Rad52 mutant proteins differ from mutant to wild type and from mutant to mutant. This is true whether the proteins are expressed from high-copy or from single-copy plasmids (Asleson et al. 1999). While some mutations greatly decrease the protein levels (Rad52-2p), intriguingly, other mutations, all within the middle region of Rad52p, increase the quantity of the protein above normal wild-type levels (Rad52-Δ210Bp, Rad52-96Ap). We went on to determine the half-lives of several mutant forms of Rad52p relative to the half-life of wild-type Rad52p. Consistent with the observation that some mutations in Rad52p increase cellular levels, we also found that both Rad52-96Ap (R235G) and Rad52-Δ210Bp increase the half-life more than twofold over the 15-min half-life of the wild-type protein. Because only mutations in this middle region of Rad52p extend the half-life of the protein and because this effect does not result from differences in expression or mRNA stability, we conclude that amino acid residues in the middle of Rad52p are important for its post-translational regulation.

We measured the half-life of wild-type Rad52p and found it to be 15 min. While we measured the half-life of Rad52p by high-level expression, we believe this value to be indicative of its cellular half-life. Using the same expression system, we had previously shown that the functional half-life of Rad52p in recombinational repair is between 15 and 30 min (Dornfeld and Livingston 1991). The short functional half-life argues that Rad52p either is turned over rapidly or must be synthesized contemporaneously with its need. Furthermore, expression of a high level of Rad52p does not alter the level of a fully functional, epitope-tagged copy of Rad52p expressed at a normal level. This experiment argues against the possibility that only excess Rad52p is degraded rapidly. The two experiments are consistent with the conclusion that the normal cellular complement of Rad52p has a half-life of ~15 min.

We have also measured the half-life of *RAD52* mRNA and all mutant forms, but does not increase the half-life of Rad52p and found it to be shorter than the protein half-life. In addition, we found no difference between the half-lives of mRNA that encode the wild-type and mutant proteins. Because selective degradation of mRNA expressed to high levels is unlikely, these measurements also are consistent with the conclusion that the measured half-life of Rad52p is short.

More important to our studies is the observation that the cellular levels of wild-type and mutant proteins expressed as single-copy sequences from the *RAD52* promoter correlate with their measured half-lives. This means that the differences in half-lives contribute to the differences in cellular levels. What is novel about our observations is that there are mutations that increase both the cellular level and the measured half-life. Most mutations weaken the stable conformation of the encoded protein and lead to more rapid degradation.

The extended half-lives of the mutant proteins with alterations in the middle region of Rad52p raise the question of whether the mutational changes increase the stability of Rad52p because they prevent modifications that act as a signal for degradation or because they disrupt a Rad52p domain essential for interaction with a protein that affects Rad52p degradation. The internal deletion proteins either could be missing residues whose modification is needed for degradation or could fail to...
interact with a protein that directs Rad52p degradation. The missense mutant with the single-amino-acid change, R235G, is less likely to be missing a residue that becomes modified and more likely to disrupt interaction with a protein that modifies adjacent residues in Rad52p. Whether the factor binding to the middle region makes modifications or simply acts to hasten Rad52p turnover, what is clear is that the middle region of Rad52p is important to its stability and is likely to contain a binding site for another protein that controls its degradation. According to this explanation, in the case of the deletion proteins, the binding site is missing, and in the case of the protein containing the missense mutation, the binding site is weakened. Therefore, we expect that overexpression of a degradative factor should decrease the half-life of Rad52-96A but not that of Rad52Δ210Bp.

By genetic means we eliminated a number of factors that might be candidates for the factor controlling Rad52p degradation. Neither changes in the amount of Rad51p nor that of Rfa2p increase the wild-type protein’s half-life or decrease the cellular level of Rad52p-96Ap. In addition, we tested mutants with deletions of the RAD59, SIR3, RAD6, and SRS2 genes, all of which have genetic or physical interactions with RAD52, and did not find alteration of the half-life of Rad52p (Table 2; Scheidt et al. 1990; Milne et al. 1995; Schild 1995; Kaytor and Livingston 1996; Nguyen and Livingston 1997a; Davis and Symington 2001). Similarly, the addition of MMS to the cultures did not change the half-life, signifying that a demand for Rad52p neither prolongs its life nor hastens its degradation (Table 2).

A mutation that alters the cellular level of wild-type Rad52p and all its mutant forms is a mutation in the proteasome pup1-1. A number of connections between the proteasomal degradation system and Rad52p, Rad51p, and RPA are known. RPA is likely degraded by the proteasome as its half-life is dependent on Rsp5p, a ubiquitin ligase (Erdeniz and Rothstein 2000). In mammalian cells both Rad51p and Rad52p have been shown to interact with the ubiquitin paralog Ubl1p (SUMO-1), the ortholog of yeast Smt3p, and with Ube2Ip, the ubiquitin-conjugating enzyme that is the ortholog to the yeast Ubc9p (Shen et al. 1996a,b). Furthermore, we selected a mutation of a protease subunit of the 20S proteasome, pup1-1, as a suppressor of a temperature-sensitive rad52 mutation (Nguyen and Livingston 1997a). While the proteasomal mutations lead to decreased cellular levels of Rad52p, we found that this is not the result of an increased half-life but rather a result of an increased cellular level of RAD52 mRNA. The increase in the cellular level is unique to RAD52 and is not shared by messages of other repair and replication proteins. This specificity, together with the observation that the RAD52 mRNA half-life does not change in a pup1-1 strain, led us to conclude that RAD52 is under a novel form of transcriptional control that is regulated by the proteasome.

The measured half-life of Rad52p is shorter than that of either Rad51p or RPA, two proteins known to interact with Rad52p. By high-level expression we measured the half-lives of RAD51 mRNA and protein and found them to be longer than the corresponding values for RAD52. Previous measurement of the half-life of RPA shows its half-life to be approximately four times the 15-min value for Rad52p. Microarray studies show that the RAD51 mRNA and the RFA mRNA encoding the three subunits of RPA are expressed strongly only at the beginning of S phase. In contrast, RAD52 is expressed at low levels throughout the cell cycle (Spellman et al. 1998). Thus, Rad52p may be unique in relation to Rad51p and RPA in that it is made continuously throughout the cell cycle and appears to have a shorter half-life than that of the other two proteins.

We hypothesize that Rad52p acts as an early signal in recombinational repair. It surveys DNA molecules for damage, carries out early functions in binding and strand exchange, and, when necessary, recruits Rad51p. Because Rad51p is not necessary in all types of recombinational repair, Rad52p probably participates in more than one type of complex, some involving Rad51p and some not. Degradation of Rad52p might act as a signal for rearrangements of complexes. For example, in Rad51p-dependent repair, Rad52p decay might permit Rad51p to bind its accessory factors, Rad54p and...
Rad55p-Rad57p, to promote the completion of recombinational repair.

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