Interaction of the yeast *RAD7* and *SIR3* proteins: implications for DNA repair and chromatin structure

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We have used the two-hybrid system to identify proteins that interact with the product of *RAD7*, a gene involved in DNA repair. A screen of a yeast genomic DNA–*GAL4* activation domain (GAD) fusion gene library allowed the isolation of plasmids containing sequences corresponding to the 3' end of the *SIR3* gene. This gene is known to be involved in the production of transcriptionally silent DNA at the cryptic mating-type cassettes and at telomeres. The cloned sequences coded for amino acids 307–979 of the Sir3 protein. A *sir3* deletion allele, constructed in an isogenic *rad7*-deletion strain, rescued approximately one-quarter of the UV sensitivity associated with the *rad7* deletion, indicating that the two genes interact genetically. Radiolabeled fusion proteins, made with the glutathione *S*-transferase (GST) gene in the vector pGEX-2T, were purified from *Escherichia coli* and shown to interact in vitro. This evidence suggests that the Sir3 protein interacts with the Rad7 protein to allow the nucleotide excision repair complex access to transcriptionally inactive chromatin. The proportions of 5-FOA-resistant cells in cultures from isogenic *RAD7*⁺ and *rad7*-Δ strains containing a telomeric *URA3* gene were similar, suggesting that the *RAD7* gene is not involved in the production or structure of transcriptionally silent chromatin at the telomeres. *RAD7*-dependent DNA repair of transcriptionally silent chromatin was shown not to induce expression of a telomeric copy of the *URA3* gene, suggesting that repair of transcriptionally silent chromatin differs from transcriptionally active chromatin. Expression of a telomeric copy of the *URA3* gene was stimulated in a *rad7*-Δ mutant, suggesting that repair of lesions in the absence of Rad7 can result in the activation of transcriptionally silenced genes.

[Key Words: *Saccharomyces cerevisiae*; DNA repair; two-hybrid system; *RAD7*; *SIR3*]

Received April 1, 1994; revised version accepted July 19, 1994.

Some 11 genes involved in nucleotide excision repair (NER) have been identified in *Saccharomyces cerevisiae* (for review, see Friedberg 1988). Mutations in a subset of genes essential for NER, *RAD1*, *RAD2*, *RAD3*, *RAD4*, *RAD10*, and *MMS19*, result in yeast strains that are highly defective in the repair of DNA damage, such as cyclobutane pyrimidine dimers (CPDs), and are extremely sensitive to the lethal effects of DNA damage (Reynolds and Friedberg 1981; Wilcox and Prakash 1981). Recent evidence from a number of laboratories has shown that the *RAD3* and *RAD25* gene products are associated with the yeast RNA polymerase II initiation factor b complex, linking them to the process of transcription (Feaver et al. 1993; Guzder et al. 1994a,b). In contrast, mutations in other genes, *RAD7*, *RAD16*, and *RAD23*, have been classified as having an accessory role in NER. These mutations cause a partial defect in the

removal of CPDs and are less sensitive to this type of DNA damage (Miller et al. 1982; Bang et al. 1992).

The *RAD7* gene was cloned by complementation of the UV-sensitive phenotype of a *rad7* mutant strain (Perozzi and Prakash 1986). The *RAD7* open reading frame (ORF) is 1695 bp in length, coding for a polypeptide of 565 amino acids with a molecular mass of 63.7 kD. The predicted amino acid sequence of the *RAD7* gene gives little information about its function in NER. However, the limited UV sensitivity of a *rad7*-deletion mutant suggested that Rad7 acts as accessory protein, perhaps required to give the NER complex access to damage in chromatin (Perozzi and Prakash 1986; Schiestl and Prakash 1989). The Rad7 protein was also found to contain a number of tandemly repeated leucine-rich motifs (LRMs) in the carboxyl terminus, which may be involved in specific protein–protein interactions, perhaps related to its function in NER (Schneider and Schweiger 1991).

In *S. cerevisiae*, transcriptionally silent chromatin is found at the *HML* and *HMR* loci, encoding the cryptic

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mating-type cassettes and at telomeres (for review, see Laurenson and Rine 1992; Sandell and Zakian 1992). Genes positioned close to, or within, these domains exhibit transcriptional repression (Klar et al. 1981; Schnell and Rine 1986; Mahoney and Broach 1989; Gottschling et al. 1990). Defects in six genes (*SIR2*, *SIR3*, *SIR4*, *NAT1*, *ARD1*, and *HHF2*) affect transcriptional silencing at *HML*, *HMR*, and telomeres (Laurenson and Rine 1992; Sandell and Zakian 1992).

The *SIR3* gene product is involved in the repression of gene expression at the level of transcription. The mode of action of Sir3 is currently unknown, but it is believed to be involved in the packaging of DNA into transcriptionally silent chromatin (see above reviews). Haploid yeast strains carrying a *sir3* mutant allele are unable to mate because of the expression of both α and a information in the same cell. The structure of chromatin at the telomere is also affected in a *sir3* mutant (Aparicio et al. 1991; Braunstein et al. 1993). It has been suggested that the *SIR3* gene product may be a structural component of transcriptionally silent chromatin in yeast, or at least responsible for its assembly. Renaud et al. (1993) suggested that Sir3 is the functional equivalent of histone H1, mediating the supranucleosomal structure of the genome.

Terleth et al. (1990) first showed that *rad16* and *rad7* yeast strains were defective in the removal of CPDs from transcriptionally silent chromatin found at *HML α* , one of the cryptic mating-type loci. Thus, the *rad7* and *rad16* mutants are presumably defective in factors important for making CPDs in transcriptionally silent chromatin accessible to the excision repair enzymes. These investigators also showed that the defect in removal of CPDs from the *HML α* locus was reversed in a *sir3* mutant strain, suggesting that changes in chromatin structure mediated by the *SIR3* gene product were involved in this process (Terleth et al. 1989).

We have used the two-hybrid system (Fields and Song 1989; Chien et al. 1991) to identify proteins that interact with *RAD7*. By use of a plasmid carrying an *Escherichia coli* *lexA*–*RAD7* fusion gene, we have screened three yeast genomic DNA–GAL4 activation domain (GAD) libraries. Here, we report the identification of a number of plasmids that stimulated the transcription of the *lacZ* reporter gene in combination with the *lexA*–*RAD7* fusion. Two of the pGAD fusion plasmids contained a carboxy-terminal fragment of the *SIR3* gene. An interaction between the Rad7 and Sir3 proteins was also demonstrated in vitro when both proteins were produced as fusions to glutathione *S*-transferase (GST) in *E. coli*. We have also shown that a deletion mutation of *sir3* shows a genetic interaction with a *rad7* deletion mutation in the same strain. These results suggest that the *RAD7* protein acts through an interaction with the *SIR3* protein. However, deletion of the *RAD7* gene had no effect on the silencing of a telomeric copy of the *URA3* gene, indicating that Rad7 is not involved in the production of silent chromatin. Furthermore, *RAD7*-dependent NER of silent chromatin at telomeres does not appear to induce transcription.

Results

Isolation of *Rad7*-interacting GAD fusion proteins

In an effort to understand the role that *RAD7* plays in nucleotide excision repair, we have used a modified version of the two-hybrid system (Fields and Song 1989; Chien et al. 1991) to identify interacting proteins that also function in this process. The complete *RAD7* ORF was cloned into the plasmid pBTM116 (kindly supplied by Dr. S. Fields, State University of New York, Stony Brook) to produce a *lexA*–*RAD7* fusion (see Materials and methods). This plasmid was transformed first into the yeast strain CTY10-5D, which was then transformed with a mixture of each of the three Gal4 activation domain libraries. Approximately 400,000 colonies were replicated to medium containing X-gal, and 7 blue colonies were isolated and purified. To determine whether the production of β -galactosidase in the blue colonies was dependent on the presence of both plasmids, the plasmid containing the *lexA*–*RAD7* fusion was lost by growth in medium selecting only for the pGAD plasmids (SC–Leu[–]). Yeast colonies containing only the pGAD fusions were tested on SC–Leu[–] medium containing X-gal for color production. Four pGAD fusions that were dependent on the presence of the *lexA*–*RAD7* fusion plasmid for β -galactosidase activity were analyzed further. They were isolated from yeast and transformed into *Escherichia coli*. The *EcoRI* digestion patterns of the four plasmids were different, suggesting that each contained a unique cloned DNA fragment (data not shown). When sequenced with a primer complementary to the Gal4 activation domain (see Materials and methods), and two plasmids, pGAD7B5 and pGAD7B34, proved to have the same DNA sequence from the fusion junction but differed in the length of fragment cloned. The DNA sequences generated from the four pGAD7B plasmids were compared for homologies with the DNA sequences in the GenBank data base by use of the FASTA program (Pearson and Lipman 1988). The sequences from pGAD7B5 and pGAD7B34 were found to be identical to a portion of the *SIR3* gene (Fig. 1A). The junction of the fusion in the *SIR3* gene occurred at an internal *Bgl*III site to give a fusion protein from amino acid 307 through to the end of the gene. Both plasmids contained fusions at the same site in the vector pDP4 (GAD-1F). The other two plasmids, pGAD7B6 and pGAD7B35, both produced fusion proteins, as determined by the DNA sequence after the fusion junction, and contained unique DNA sequences not found in GenBank.

The plasmid pGAD7B5, containing the GAD–*SIR3* fusion, was then transformed back into the yeast strain CTY10-5D containing the *lexA*–*RAD7* fusion and also CTY10-5D containing the *lexA*–*RAD6* gene fusion plasmid. The production of β -galactosidase activity was found to be dependent on the presence of both pGAD7B5 (GAD–*SIR3*) and pBTM116–*RAD7* (*lexA*–*RAD7*) (see Table 1). Activity of *RAD7*–*SIR3*-stimulated β -galactosidase was 23-fold higher than in the *SNF1*–*SNF4*-positive control. Typically the *RAD7*–*SIR3* yeast colonies turned blue on X-gal medium after an overnight incubation at

Figure 1. Summary of *SIR3* deletion map of Rad7-interacting domain. (A) The entire *SIR3* gene (2937 bp) along with the amino- and carboxy-terminal deletions is shown with the first and last amino acid number indicated at the boundaries. The restriction enzymes shown are those used to generate deletions. The plasmid designation is listed at left. Restriction sites are designated by the following letters: (Bg) *Bgl*II; (E) *Eco*RI; (H) *Hin*dIII; (E II) *Bst*II; (B I) *Bst*BI; (N) *Nru*I; (A) *Asp*718; (X) *Xho*I. The relative β -galactosidase activity was assessed by eye, in comparison with the original *RAD7-SIR3* positives, after growth for 1–2 days at 30°C on medium containing X-gal. (B) The smallest peptide capable of *lacZ* activation when fused to the Gal4 activation domain was characterized for hydropathy. The *Bst*BI–*Eco*RI fragment containing the amino acids 687–971 of the *SIR3* gene was analyzed by use of standard Kyte–Doolittle hydropathy values. The positions of the *Nru*I (N) and *Asp*718 (A) restriction sites are noted.

30°C, whereas the *SNF1-SNF4* interaction usually required 3 days for the first noticeable color development, suggesting that the interactions between the Rad7 and Sir3 portions of the fusion proteins are strong. Clearly the interaction between *lexA-RAD7* and *GAL4_{AD}-SIR3* fusion proteins is not the result of nonspecific interactions of the type described by Bartel et al. (1993).

Deletion mapping of the Sir3-interacting domain

The *SIR3* protein is 979 amino acids in length with a predicted molecular mass of 111,207 daltons. The *GAL4_{AD}-SIR3* fusion in pGAD7B5 was fused at the *Bgl*II site at nucleotide position 916 of the ORF, which corresponds to amino acid 307 (Fig. 1A). The location of the

specific domain that interacts with Rad7 was mapped by amino- and carboxy-terminal deletion analysis of a *SIR3* DNA fragment in the *GAL4_{AD}* fusion plasmid pACTII (see Fig. 1A). The interacting domain appears to be contained within the last one-third of the ORF, beginning at the *Bst*BI site at nucleotide 2055 and amino acid 687, and ending with the *Eco*RI site at nucleotide position 2911 and amino acid 971, 8 amino acids short of the stop codon. The carboxy-terminal deletion bounded by the *Asp*718 site completely removes the ability of the Sir3 fusion to interact with the *lexA-Rad7* fusion. This puts the carboxy-terminal boundary of the interacting domain between the *Asp*718 site at amino acid 835 and the *Eco*RI site at amino acid 971. The method of Kyte and Doolittle (1982) was used to plot the hydropathic index of the amino acid sequence of the identified interacting

Table 1. Transcriptional activation of hybrid proteins

Yeast transformant		β -Galactosidase activity ^a
DNA-binding domain hybrid	activation domain hybrid	
<i>lexA</i> (1–202)– <i>RAD7</i> (1–565)	—	<1
—	<i>GAL4</i> (768–881)– <i>SIR3</i> (307–978)	<1
<i>lexA</i> (1–202)– <i>RAD7</i> (1–565)	<i>GAL4</i> (768–881)– <i>SIR3</i> (307–978)	414
<i>lexA</i> (1–202)– <i>RAD6</i> (1–171)	<i>GAL4</i> (768–881)– <i>SIR3</i> (307–978)	<1
<i>GAL4</i> (1–147)– <i>SNF1</i>	<i>GAL4</i> (768–881)– <i>SNF4</i>	18

^aExpressed in Miller units (see Materials and methods). The values are averages from three separate transformants each assayed in duplicate.

domain of *SIR3*. The results in Figure 1B show a hydrophobic region between amino acids 150 and 280 (835–971 of the complete Sir3 product), which could be involved in the interaction with Rad7.

Rad7 and Sir3 fusion proteins interact in vitro

To confirm that the interaction between the Rad7 and Sir3 proteins occurs at the protein level, we produced GST fusion proteins in *E. coli* with both the Rad7 protein and a carboxy-terminal portion of the Sir3 protein. This was accomplished by cloning the entire *RAD7* ORF in-frame into the vector pGEX-2T. The *Bgl*II–*Bam*HI fragment containing the *SIR3* gene sequence was cloned into the *Bam*HI site of the same vector. Both plasmids were transformed into the *E. coli* strain JM109. Transformants produced GST fusions when induced with IPTG. Maximum levels of fusion proteins were obtained following growth at 23°C in LB containing 1 M sorbitol and 2.5 mM betaine. This suggests that fusion protein yield is being affected by problems with protein folding (Blackwell and Horgan 1991).

Radiolabeled fusion proteins were produced in M9 medium containing 1 M sorbitol and 2.5 mM betaine and 150 μ Ci of [35 S]methionine. Both labeled and unlabeled fusion proteins were purified by affinity chromatography with glutathione–agarose (GA) beads. To test whether the two proteins could interact in vitro, the radiolabeled fusion proteins were first cleaved from the GST portion of the proteins by thrombin protease digestion, which cuts at a thrombin cleavage site at the fusion protein junction. This liberated the Rad7 and Sir3 portions of the fusion proteins from the GA beads into the soluble fraction, referred to as thrombin-cut supernatant (TCS). The interaction between the Rad7 and the Sir3 proteins was tested by mixing (1) the [35 S] methionine-labeled Rad7 TCS with unlabeled GST–Sir3 fusion protein bound to GA beads and (2) [35 S] methionine Sir3 TCS with unlabeled GST–Rad7 bound to GA beads. Figure 2 shows that in both experiments radiolabeled peptides of the expected size for the thrombin-released peptides were associated with GA beads containing fusion protein of the other type. Lane C contains the radiolabeled proteins from the GST–Sir3 TCS bound to the GA beads containing nonlabeled GST–Rad7 protein. The peptide fragment of Sir3 released should be 76 kD, which corresponds to the size of the band observed. The reciprocal experiment, lane D, shows a band at ~71 kD, 7 kD larger than expected for Rad7. This is likely a property of the Rad7 proteins as the GST–Rad7 fusion protein also tends to migrate slower than expected. The estimated size of the GST–Rad7 fusion protein from a 10% SDS–polyacrylamide gel is 97 kD, also ~7 kD over the predicted size from the protein sequences. As these values are close, it appears that Rad7 migrates slower than expected during SDS-PAGE. Lanes B and E show that these radiolabeled proteins are not bound by the GA beads alone. These results demonstrate that the Rad7 and Sir3 proteins appear to interact directly.

Figure 2. Rad7 and Sir3 proteins produced as fusions to GST interact in vitro. The Rad7 protein and a Sir3 peptide (amino acids 307–978) were radiolabeled and produced as fusions to GST in *E. coli*. These proteins were denatured and electrophoresed on a 10% SDS PAGE gel and fluorographed for 3 days at –80°C. (Lane A) Thrombin-cut Sir3 supernatant; (lane B) thrombin-cut Sir3 supernatant mixed with glutathione beads; (lane C) thrombin-cut Sir3 supernatant mixed with GST–Rad7 glutathione–agarose beads; (lane D) thrombin-cut Rad7 supernatant mixed with GST–Sir3 glutathione–agarose beads; (lane E) thrombin-cut Rad7 supernatant mixed with glutathione–agarose beads (lane F) thrombin-cut Rad7 supernatant. The arrows labeled Sir3 and Rad7 indicate the position of the thrombin-digested peptides.

Deletion of the SIR3 gene affects RAD7-dependent DNA repair

If the interaction between Rad7 and Sir3 has biological significance we might expect that a mutation in the *SIR3* gene would affect the DNA repair phenotype of a *rad7* mutant. To test this, we constructed a set of isogenic yeast strains, containing *rad7-Δ*, *sir3-Δ*, or *rad7-Δ-sir3-Δ* deletions and determined their survival rates after exposure to UV light. The results are shown in Figure 3A. *RAD*⁺ and *sir3-Δ* strains were relatively resistant to UV. However the *rad7-Δ* strain was about four orders of magnitude more sensitive at a dose of 60 J/m². Interestingly, the *rad7-Δ-sir3-Δ* strain was significantly less sensitive than the *rad7-Δ* strain. It also appeared that the *sir3-Δ* strain was more resistant to UV than the *RAD*⁺. We next investigated the effect of the *sir3-Δ* deletion on the UV resistance of other DNA repair mutants. The double mutants *sir3-Δ-rad1-Δ* and *sir3-Δ-rad6-Δ* were both as sensitive as *rad1-Δ* and *rad6-Δ* mutants. The results showing *sir3-Δ* in combination with *rad1-Δ* and *rad6-Δ* are found in Figure 3 (B,C). This suggests that the genetic interaction between the *sir3-Δ* and the *rad7-Δ* deletions is not the result of a general increase in UV resistance, resulting from the loss of *SIR3* function, but rather a specific interaction between the two gene products.

RAD7 is not involved in silent chromatin formation

The *SIR3* protein is an important component of silent chromatin production at both the cryptic mating-type

Figure 3. The *sir3-Δ* mutation rescues part of the UV sensitivity of the *rad7-Δ* mutation. Isogenic strains carrying a *rad7-Δ*, *sir3-Δ*, *rad1-Δ*, *rad6-Δ* or double mutants *rad-Δ sir3-Δ* were assayed for their UV sensitivity as described in Materials and methods. Four individual colonies from each strain were assayed, and the results were averaged and plotted against the dose. Note: Because the differences in UV sensitivity between *RAD*⁺ and *sir3-Δ* are negligible, the survival curves are superimposed. (A) (Δ) *RAD*⁺; (□) *rad7-Δ*; (■) *sir3-Δ*; (▲) *rad7-Δ sir3-Δ*. (B) (Δ) *RAD*⁺; (○) *rad1-Δ*; (■) *sir3-Δ*; (●) *rad1-Δ sir3-Δ*. (C) (Δ) *RAD*⁺; (▽) *rad6-Δ*; (■) *sir3-Δ*; (▼) *rad6-Δ sir3-Δ*.

loci and at telomeres (Aparicio et al. 1991; Braunstein et al. 1993). The interaction between Rad7 and Sir3 suggested that Rad7 might also be involved in silent chromatin structure. To test this possibility we measured the production of 5-FOA-resistant yeast cells in *RAD*⁺ and *rad7-Δ* yeast strains containing a copy of the *URA3* gene integrated at telomere VII L. 5-FOA is lethal to yeast cells expressing the *URA3* gene (Boeke et al. 1987). This allows the quantitation of telomere position effect (TPE) silencing by measuring the fraction of 5-FOA-resistant colonies in a population of cells (Gottschling et al. 1990). The ability of a cell to give rise to a colony on 5-FOA indicates that the cell contains little or no *URA3* gene product when plated. Normally, between 10% and 90% of the cells in a culture containing a telomeric *URA3* gene are 5-FOA resistant when grown on medium not requiring *URA3* function (Gottschling et al. 1990). Mutations in genes important for silent chromatin structure

decrease the proportion of 5-FOA-resistant cells. Typically, *sir* mutants give rise to 5-FOA-resistant cells with a frequency of $<1.8 \times 10^{-7}$ (Aparicio et al. 1991). If Rad7 is involved in the structure or production of silent chromatin at telomeres, one might expect a dramatic reduction in the numbers of 5-FOA-resistant cells in the *rad7-Δ* strain compared with a *RAD*⁺ strain. This was clearly not the case. The fraction of 5-FOA-resistant cells was determined for 10 individual colonies of each of the isogenic strains UCC1001 (*RAD*⁺) and UCC1083 (*rad7-Δ*), after growth for ~10 generations in YPAD medium. The wild-type strain (UCC1001) produced a typical fraction of 0.42 of 5-FOA-resistant cells in each culture with a range from 0.21 to 0.86. The *rad7-Δ* strain (UCC1083) produced a similar result, median 0.49 (range 0.13–0.89), suggesting that the Rad7 protein does not take part in the production or the structure of silent chromatin at telomeres.

Expression of URA3 in silent chromatin is stimulated in rad7-Δ but not in RAD⁺

The removal of CPDs from transcriptionally silent chromatin, such as the HM loci or telomeres, likely requires some form of chromatin restructuring; possibly conversion to a form similar to that found in transcribed regions of the genome. Recently, Aparicio and Gottschling (1994) showed that if the *URA3* trans-activator PPR1 was present in high enough concentrations at an appropriate time in the cell cycle it could overcome silencing at telomeres. This suggests that, if the chromatin structure of a silenced telomeric *URA3* were remodeled to allow DNA repair, one might expect to see an increase in the expression of this gene concomitant with an increase in DNA damage. If UV-induced DNA damage to the silent telomeric copy of *URA3* is repaired without stimulation of gene expression, as a result of chromatin remodeling, then the survival curves on YPAD and 5-FOA medium should be identical. If expression of the silenced *URA3* gene is induced, then those cells in which this occurs will be killed on FOA medium resulting in a lower relative survival rate than on YPAD. To test this possibility, the yeast strains UCC1001 (*RAD*⁺) and UCC1083 (*rad7-Δ*) were grown overnight in YPAD. Four independent cultures of each strain were harvested, washed and resuspended in sterile water, and irradiated with UV. Appropriate dilutions were plated onto YPAD and 5-FOA medium and incubated at 30°C in the dark until colonies appeared. Because telomere silencing within a culture is not absolute, the number of colony forming units (cfus) on 5-FOA medium are always lower than on YPAD. Accordingly, the relative survival rates at each dose were calculated from the number of cfus on the nonirradiated plates of each type of medium.

The mean survival at each dose on YPAD and 5-FOA was determined for the four cultures of UCC1001 (*RAD*⁺) and UCC1083 (*rad7-Δ*) and tested for significant differences by use of the two sample *t*-test. The means obtained for UCC1083 (*rad7-Δ*) on YPAD versus 5-FOA differed significantly at the 5% level for the 10 J/m²

dose and at the 1% level for the 20 J/m² and 40 Joules/m² doses. The results for both strains are plotted in Figure 4. The *rad7-Δ* mutation reduces the number of FOA^R cells sixfold at 40 J/m², suggesting that DNA repair of telomeric chromatin in the absence of Rad7 stimulates expression of a silenced *URA3* gene.

Discussion

We have used the two-hybrid system to identify a protein-protein interaction between the products of *RAD7*, a gene involved in DNA repair, and *SIR3*, a gene involved in transcription silencing. Both the library fusion plasmids pGAD7B5 and pGAD7B34 and the *lexA-RAD7* plasmid are required for the stimulation of β-galactosidase from the *lacZ* reporter gene construct. β-Galactosidase is not produced by the combination of the pGAD7B5 and *lexA-RAD6* plasmids. These observations suggest that the interaction between Rad7 and Sir3 is not a false positive of the type described by Bartel et al. (1993). It is possible that the interaction could be mediated by a third-party protein, which could either be an artifact or a real *in vivo* activity; however, the demonstration that the Rad7 and Sir3 proteins interact *in vitro* suggests that the phenomenon is real and has biological significance in yeast.

The observation that a *sir3* deletion reduces the UV sensitivity of a *rad7* deletion demonstrates that both proteins are involved, in some way, in the repair of transcriptionally silent chromatin. As Sir3 is likely a component of silent chromatin (Renauld et al. 1993), Rad7 possibly acts to remodel the structure of silent chromatin, allowing the NER complex access to the CPDs in

this region of the genome. Certain *sir3* mutations are able to suppress a defect in transcriptional silencing because of a histone H4 mutation, suggesting that Sir3 interacts either with histone H4 or with the nucleosome (Johnson et al. 1990). The mutations in *SIR3* that suppress histone H4 mutants alter amino acids 86 and 205; however, the interaction with Rad7 involves a different region, spanning amino acids 687–971. There is also evidence that Sir3 may interact directly with Rap1, an essential transcriptional regulator involved in telomere length maintenance and telomeric silencing in yeast (P. Moretti, K. Freeman, and D. Shore, cited in Palladino et al. 1993). The Rad7 protein could be involved in the mediation of either of these proposed Sir3 interactions in response to DNA damage. This could then allow the remodeling of silent chromatin and repair of CPDs to occur in this region of the genome. Because only about one-quarter to one-third of the UV sensitivity of the *rad7* deletion mutant is rescued by deletion of *SIR3*, there may be other proteins involved in chromatin structure that are an impediment to repair in a *rad7* deletion strain. Alternatively, Rad7 may interact with other repair proteins to produce some repair activity important to the repair of CPDs in other regions of the genome. Recently, Verhage et al. (1994) have shown that deletion mutants of *RAD7* and *RAD16* are defective in the repair of DNA damage found in the nontranscribed strand of a transcriptionally active gene. This finding shows that *RAD7* is involved in the repair of transcriptionally silent chromatin found at the HM loci and at telomeres as well as the repair of lesions found in transcriptionally active genes. This newly described function of *RAD7* is likely responsible for the remaining UV sensitivity seen in a *rad7-Δ sir3-Δ* yeast strain.

The two other plasmids, pGAD7B6 and pGAD7B35, contain DNA sequences that code for products that interact with *RAD7* in the two-hybrid system and that may represent additional genes involved in repair of the silent chromatin and/or the nontranscribed strands of active genes. These plasmids are being analyzed to determine the involvement of the cloned sequences in *RAD7*-dependent NER.

It is interesting that the Rad7–Sir3 interaction that occurs in the two-hybrid system and also with the GST fusion proteins *in vitro* does so in the absence of DNA damage. This could suggest that the interaction occurs *in vivo* without the need for DNA damage and that the Rad7 and Sir3 proteins may be normally associated with each other in transcriptionally silent chromatin. If so, it is possible that Rad7 is involved in the production or structure of silent chromatin. We have shown that deletion mutants of *RAD7* have no effect on the proportion of 5-FOA-resistant cells in a yeast strain with a telomeric copy of the *URA3* gene. This is in contrast to the severe decrease in frequency of 5-FOA-resistant colonies in a *sir3* mutant strain with a similar *URA3* construct (Aparicio et al. 1991). These results suggest that Rad7 is involved in the repair of DNA damage to transcriptionally silent chromatin but is not involved in the production or structure of transcriptionally silent chromatin. This

Figure 4. Repair of CPDs in silent chromatin does not stimulate transcription. Four cultures of the isogenic yeast strains UCC1001 (*RAD*⁺) and UCC1083 (*rad7-Δ*), each with a copy of the *URA3* gene integrated at the telomere of chromosome VII L, were assayed for survival on YPAD and 5-FOA medium. The means of the survival at each dose were calculated as described in Materials and Methods and plotted as the log of the percent survivors vs. the UV dose in J/m². (■) UCC1001 (*RAD*⁺) YPAD survival curve; (△) UCC1001 (*RAD*⁺) 5-FOA survival curve; (□) UCC1083 (*rad7-Δ*) YPAD survival curve; (▲) UCC1083 (*rad7-Δ*) 5-FOA survival curve.

raises the possibility that Rad7 and Sir3 do not interact in the absence of DNA damage. It is likely that Sir3 normally interacts with other proteins, possibly histone H4 and/or Rap1, to allow the formation of transcriptionally silent chromatin. Following UV irradiation, Rad7 interacts with Sir3, displacing the other proteins and facilitating access of the NER complex to the damaged DNA.

The NER genes in yeast are constitutive but weakly expressed (Hoeijmakers 1993); in addition, *RAD7* shows a sixfold increase in transcript level in response to UV (Jones et al. 1990). Sir3 is normally present at a low level in the cell at all times (Ivy et al. 1986). The interaction observed in the two-hybrid system in the absence of DNA damage is likely a consequence of the overproduction of the Gal4_{BD}-Rad7 and Gal4_{AD}-Sir3 fusion proteins. Because any proteins that interact with Rad7 or Sir3 will be produced at normal levels in the two-hybrid yeast strain this overproduction would result in an interaction between the excess Gal4_{BD}-Rad7 and Gal4_{AD}-Sir3 fusion proteins in the absence of DNA damage.

The experiments utilizing a yeast strain containing a telomeric *URA3* gene showed that DNA repair of transcriptionally silent chromatin does not stimulate transcription of silenced genes (UCC1001 in Fig. 4). This suggests that the changes necessary to permit NER in silent chromatin are not equivalent to the chromatin structure found in transcriptionally active areas of the genome. Thus, the NER of transcriptionally silent DNA is a process that is different from the conversion to transcriptionally competent chromatin. In the isogenic *rad7* deletion strain survival on 5-FOA medium was reduced after UV irradiation. We attribute this to an increase in the proportion of cells in which the telomeric copy of *URA3* was expressed because of the stimulation of transcription of this gene following repair of UV-induced damage. For a UV-irradiated yeast cell to survive and form a colony, all potentially lethal DNA lesions must be repaired by one of the known repair systems. DNA repair of silenced chromatin must still occur in the absence of Rad7 but by a mechanism that does not require this gene product. There is evidence that the *RAD7*- and *RAD6*-dependent repair systems compete for the same substrate (Schiestl and Prakash 1989). *RAD6* is involved in postreplication repair (Prakash 1981). It is likely that this repair system is responsible for dealing with some of the UV-induced lesions in a *rad7*-Δ strain and results in the expression of the silenced telomeric copy of *URA3*. It will be interesting to determine which other DNA repair genes are involved in this effect.

We propose the following model to account for our findings. Rad7 is produced constitutively and functions both in the repair of DNA damage in the nontranscribed strands of active genes and in transcriptionally silent chromatin. The interaction between Rad7 and Sir3 is directly involved in facilitating the access of the NER complex to DNA damage in transcriptionally silent chromatin. Sir3 interacts with a number of proteins, probably including histone H4 and Rap1, to maintain the structure of silent chromatin. UV damage induces tran-

scription of *RAD7*. The higher levels of the Rad7 protein result in increased interactions with Sir3, displacing the other proteins involved in the maintenance of silent chromatin structure. This results in access of the NER complex to the DNA damage sites in silent chromatin but does not allow transcription of genes in these regions. After repair is complete, Rad7 dissociates from Sir3, and the silent chromatin domains are reconstituted. In the absence of Rad7, other DNA repair systems handle the damage found in silent chromatin domains; however this secondary repair process may result in the activation of transcription of genes in these domains.

We have demonstrated that the two-hybrid system is a valuable tool for the investigation of protein interactions involved in NER. We are continuing our efforts to identify additional genes and understand the mechanisms of NER in transcriptionally silent chromatin.

Materials and methods

Strains and DNAs

Growth and manipulation of yeast strains were done according to standard methods (Sherman 1991). All two-hybrid system experiments were done with either the strain CTY10-5D [*MATa ade2 gal4 gal80 his3-Δ200 leu2-3,112 trp1-Δ901 URA3-lexA op GAL1 → lacZ*] or *GGY1-171 [leu2-3,112 his3-Δ200 gal4-Δ gal80-Δ-pRY171 (GAL1 → lacZ)]* (Chien et al. 1991). New Gal4 activation domain plasmids were constructed from pGAD1, 2, and 3 by removal of the 1.5-kb *SphI* DNA fragment containing the Gal4 activation domain. This was ligated into *SphI*-digested YEplac181 (Gietz and Sugino 1988) lacking the *BamHI* site in the multicloning site to create pDP4, pDP7 and pDP12, respectively. Libraries, with a complexity $>1 \times 10^6$ clones, were constructed in each of the three *BamHI*-digested pDP vectors. Genomic DNA from a strain carrying a *gal4* deletion was digested with *MboI* and 3- to 5-kb fragments isolated, following electrophoresis, by the method of Girvitz et al. (1980). The fragments were ligated into the vectors pDP4, pDP7, pDP12 that had been digested previously with *BamHI* and treated with calf intestinal alkaline phosphatase (Boehringer Mannheim). Plasmid pool DNA was isolated from the *E. coli* host DH5α (*F*⁻, *recA1*, *endA1*, *gyrA96*, *thi*, *hsdR17*, *supE44*, *relA1*, *Δ[argF-lacZYA]-U169[φ80dlacZΔM15]λ*⁻) by use of the method of Birnboim and Doly (1979).

The *lexA-RAD7* fusion in plasmid pBTM116 (a generous gift from Dr. S. Fields) was made with a DNA fragment of *RAD7* amplified with the primers 5'-CGCGAATTCATGTATCG-CAGTAGAAACCGA-3' and 5'-CGCGAATTCCTTATATAC-TGTCACCTCTGTCT-3' and the polymerase chain reaction (PCR) (Innis et al. 1990) with the plasmid pGP4 containing the wild-type *RAD7* gene as a template (Perozzi and Prakash 1986). The resulting DNA fragment was purified from an agarose gel by use of the method of Girvitz et al. (1980), digested with *EcoRI*, and ligated to pBTM116 that had also been digested with *EcoRI*. The plasmid pDG649 contained the entire *RAD7* ORF in frame with the *E. coli lexA* gene. This plasmid was transformed into CTY10-5D by use of the method of Gietz et al. (1992).

All yeast gene deletions were made by use of the one-step gene disruption method of Rothstein (1983). The *SIR3* gene deletion was performed with a plasmid constructed as follows: The ~7-kb *BamHI* fragment from the plasmid pHR45-15 (kindly supplied by Dr. D. Gottschling, University of Chicago, IL) was ligated into a derivative of pUC9 containing only *BamHI*

and *EcoRI* sites, to give the plasmid pDP97. The internal *BglII*–*XhoI* fragments of *Sir3* were removed and replaced, by blunt-end ligation, with an allele of *LEU2* lacking the *EcoRI* and *KpnI* sites (Gietz and Sugino 1988) producing pDP100. This plasmid was digested with *EcoRI* and then introduced into the yeast strain MKP^o (*MATa*, *can1-100*, *ade2-1*, *lys2-1*, *ura3-52*, *leu2-3,112*, *his3-Δ200*, *trp1-Δ901*; Pierce et al. 1987), and transformants were selected on SC – Leu[–] medium. Transformants were test crossed with the strain XS803-3A (*MATa*, *leu2-3,112*, *ura3-52*, *his1-1*, *trp2*) (Schiestl et al. 1990) on YPAD medium and replicated to SC – His[–] or SC – Trp[–] medium. Failure to grow on the selection medium indicated a failure to mate and complement auxotrophic markers and was taken to indicate the *sir3-Δ* phenotype.

The *rad7* deletion was created in the strain MKP^o with the plasmid pDG79 (Schiestl and Prakash 1989), which removes nucleotides +214 to +1455 between the *XhoI* and *HindIII* sites of the *RAD7* gene and replaces it with the 1.1-kb *HindIII* fragment containing the *URA3* gene. The *RAD7* gene in strain UCC1001 was deleted by use of the plasmid pDG759. It was constructed from plasmid pDG79 (Schiestl and Prakash 1989), by cloning the entire *EcoRI* fragment into pUC8 to create pDG751. This plasmid was digested with *Asp718* and *NruI*, which cuts at positions +122 and +1824 (126 bp past the ORF) in the *RAD7* gene, removing the *URA3* gene. A *BamHI*–*EcoRI* fragment containing the *EcoRI* minus, *KpnI* minus, allele of the *LEU2* gene (Gietz and Sugino 1988), from plasmid pDG317, was ligated between these sites, (with the Klenow polymerase used to form blunt ends), to create plasmid pDG759. The *rad7-Δ* in strain UCC1083 was confirmed by Southern blot analysis (D. Gottschling, pers. comm.).

The *rad6* deletion was created by use of the plasmid pDG315 (Kang et al. 1992), which removes the entire ORF of the *RAD6* gene and replaces it with a *HpaI*–*AccI* DNA fragment containing the *LEU2* gene. The *rad1* deletion was created by transformation of the plasmid pDG18, which contains the *BamHI* DNA fragment from pRR27 (Schiestl and Prakash 1988), which contains the 1.1-kb *HindIII* *URA3* gene fragment replacing the *RAD1* DNA from –212 to +3853. All gene deletions were verified by Southern blot analysis (data not shown).

The identification of the RAD7–SIR3 interaction by use of the two-hybrid system

The yeast strain GGY1–171 containing the *GAL1* → *lacZ* reporter construct and the plasmid pDG649 (*lexA*–*RAD7* fusion) was transformed with a mixture of equal quantities of the three pDP(*GAD*) plasmid libraries by use of the method Gietz et al. (1992). Transformants (~2000–5000 per plate) were selected on SC – Trp[–] Leu[–] medium at 30°C. The colonies were grown for 3–5 days, replicated onto SC – Trp[–] Leu[–] containing 2% sucrose plus X-gal, and scored for β-galactosidase activity after an additional 1–5 days of incubation. Colonies turning blue were identified from the replicas, purified by streaking onto SC – Trp[–] Leu[–] medium, and retested on plates containing X-gal. Positives were picked from the SC – Trp[–] Leu[–] plates and grown overnight in 2 ml of SC – Leu[–] liquid medium. These cultures were then plated onto SC – Leu[–] plates to give ~250 colonies per plate and incubated at 30°C for 2 days, and the colonies were replicated to SC – Trp[–] Leu[–] plates. Those colonies containing only the pGAD plasmid (Leu⁺) were inoculated into SC – Leu[–] liquid medium and grown overnight at 30°C. DNA was prepared from these yeast cells following the method of Hoffman and Winston (1987), electroporated into electrocompetent DH5α *E. coli* by use of the Gene Pulser (Bio-Rad) with Pulse controller (25 μF, 400 Ω, 12.5 kV/cm field

strength), and Amp^R transformants isolated. Plasmid DNA was prepared from at least five individual DH5α transformants, and the specificity of the interaction with the *lexA*–*RAD7* fusion was retested by transformation back into yeast strains (CTY10–5D) lacking or containing pDG649 (*lexA*–*RAD7* fusion). Clones confirming the *GAL1* → *lacZ* activation phenotype were sequenced by use of the Sequenase kit (U.S. Biochemical) with the *GAL4* activation domain (*GAD*) primer 5′-GAAGATAC-CCCACCAAAC-3′. The GenBank data base was screened for sequences showing homology to the pGAD fusions by use of the FASTA program (Pearson and Lipman 1988).

Construction of two-hybrid system SIR3 deletion plasmids

The 1.65-kb *EcoRI* fragment containing nucleotides +1313 to +2911 of the *SIR3* ORF was cut from the plasmid pGAD7B5 and cloned into the *EcoRI* site of the plasmid pACTII (Durfee et al. 1993). The multicloning site of pACTII includes *NcoI*, *SmaI*, *BamHI*, *EcoRI*, and *XhoI* sites, and the sequence and frame is GCC ATG GAG GCC CCG GGG ATC CGA ATT CGA AGC TCG AGA. This fuses *SIR3* in-frame with the *GAL4* activation domain. Amino- and carboxy-terminal deletions were produced by partial digestion with the enzymes *BstBI*, *NruI*, *Asp718* (Boehringer Mannheim) followed by isolation of the single-cut DNA fragment from an agarose gel by use of the method of Girvitz et al. (1980). Amino-terminal deletions were produced by digestion of this fragment with a second restriction enzyme such that the product when blunt-end ligated to the first site would create an in-frame deletion. The amino-terminal deletions were made as follows; *BstBI* site ligated to the blunted *XmaI* site of pACTII, *NruI* site ligated to a blunted *XmaI* site, *Asp718* site ligated to the blunted *NcoI* site. The carboxy-terminal deletions were all made to the blunted *XhoI* site from the pACTII multicloning site, which is situated 5 bp downstream from the *EcoRI* site. The double-cut DNA fragments of the correct size were purified from an agarose gel. The 5′ overhangs of restriction fragments were treated with Klenow polymerase to produce blunt ends. Ligations were carried out under conditions that favored circularization. The ligations were electroporated into the *E. coli* strain DH5α, following the procedure of Dower et al. (1988) to allow for recovery of the appropriate plasmids. These plasmids were transformed into the yeast strain CTY10–5D containing the *lexA*–*RAD7* fusion plasmid. Blue color production was assessed after growth on medium containing X-gal for at least 24 hr.

Measurement of yeast strain UV sensitivity

Isogenic derivatives of the strain MKP^o (Pierce et al. 1987), containing the appropriate deletions for either *RAD7*, *SIR3*, *RAD1*, *RAD6*, and various combinations of *sir3-Δ* with the different *rad-Δ*, were grown overnight in liquid YPAD medium. The cell titer was determined and an appropriate number of cells plated onto YPAD plates. The plates were then irradiated in the dark with 254-nm UV light by use of a General Electric G15T8 15 W germicidal lamp at a dose rate of 1 or 2 J/m² per sec, as determined by a UVX radiometer with a model UVX-25 probe (UV Products, Inc.) The plates were incubated in the dark for 2–3 days at 30°C to determine survival rates. In experiments with strains containing a *URA3* gene integrated at a telomere, yeast cells were irradiated in suspension in water, plated, and incubated in the dark to determine survival. The percentage survival at each dose was converted to degrees by an arcsin transformation and the differences between the mean survival on YPAD versus 5-FOA for each strain were tested for significance by use of the paired *t*-test in Quattro Pro for Windows v. 5.0.

Measurement of telomere position effect

The frequency of 5-FOA-resistant yeast cells was determined as described by Aparicio et al. (1991). The strains UCC1001 (*MAT a ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1 adh4-URA3-TEL-VIII*) and its *rad7-Δ* derivative, UCC1083 (*MAT a ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-gD200 leu2-Δ1 adh4-URA3-TEL-VIII rad7Δ-LEU2*), were kindly supplied by Dr. D. Gottschling.

Production of Rad7 and Sir3 GST-fusion proteins in *E. coli*

Gene fusions to the GST gene were constructed in the vector pGEX-2T, and fusion proteins were produced in *E. coli* according to the methods of Smith and Johnson (1988). The *RAD7* *EcoRI* DNA fragment and a *SIR3* *BglII-BamHI* DNA fragment carrying amino acids 307–978 were blunt-end ligated into *XmaI* digested pGEX-2T, producing in-frame GST fusions in the plasmids pDPI04 and pDPI07, respectively. These plasmids were transformed into *E. coli* strain JM109 by electroporation, and single colonies from each transformation were grown overnight at 37°C in liquid LB plus ampicillin (50 µg/ml). These cultures were inoculated into 25 ml of LB containing ampicillin (50 µg/ml), 1 M Sorbitol, and 2.5 mM betaine (Blackwell and Horgan 1991) and were grown until an OD₆₀₀ of 1.0 was reached, at which time IPTG was added to 0.5 mM. The culture was incubated an additional 2 hr at 25°C, and then the cells were harvested via centrifugation, washed with PBS (phosphate buffered saline), and resuspended in 1 ml of PBS, 10 mM EDTA + 10 µg/ml aprotinin, 10 µg/ml leupeptin, 2 mM PMSF, and 0.1% Triton X-100 (GST buffer). The cells were lysed by sonication for two 30-sec pulses, and the lysate was cleared in a microcentrifuge for 5 min at 13,000g. The GST fusion proteins were isolated from the cleared lysate by the addition of 125 µl of a 1:1 slurry of glutathione-agarose (GA) beads in GST buffer and incubation with shaking for 15 min at 4°C. The pelleted GA beads containing GST fusion protein were rinsed twice with 1 ml of PBS and resuspended in 1 volume of PBS.

GST fusion proteins labeled with [³⁵S]methionine were produced as follows: Cells from a 25-ml culture were washed three times with 20 ml of M9 medium minus methionine containing ampicillin (50 µg/ml), 1 M sorbitol, and 2.5 mM betaine and resuspended in 1 ml of the same medium, containing 150 µCi of [³⁵S]methionine and 0.5 mM IPTG. The cells were then incubated for 2 hr at 25°C, washed twice with PBS, and lysed as above. The radiolabeled GST fusion proteins were purified and digested with the protease thrombin to release the Rad7 and Sir3 peptides into the supernatant. The GA beads were rinsed once with PBS containing BSA (10 µg/ml), twice with thrombin buffer (10 mM Tris-HCl at pH 8.0, 10 mM CaCl₂), resuspended in 100 µl of the same buffer containing 0.25 units of thrombin, and incubated at 25°C for 2 hr. The reaction was terminated by the addition of EDTA to 20 mM and Triton X-100 to 1%, and the TCS removed and pooled with 100 µl of the PBS EDTA used to rinse the thrombin-cut beads. The Rad7 TCS, 80 µl was mixed with 50 µl of a 1:1 slurry of GA beads containing GST–Sir3, and 80 µl of the Sir3 TCS was mixed with 50 µl of 1:1 slurry of GA beads containing GST–Rad7. The mixtures were incubated on ice for 2 hr, and then the GA beads were washed three times with PBS containing 20 mM EDTA, 0.1% Triton X-100, and 50 µg/ml BSA. The GA beads were then mixed with 50 µl of SDS loading buffer, heated to 95°C for 5 min, and electrophoresed on a 10% acrylamide SDS-PAGE gel. The gel was fixed with 10% acetic acid for 1 hr, washed with 20 volumes of water for 1 hr, treated with 1.0 M Na salicylate (Chamberlain 1979) for 30 min, and dried with a Bio-Rad vacuum gel dryer.

Other methods

All *E. coli* methods were performed as described in Maniatis et al. (1982). Restriction enzymes were purchased from either GIBCO BRL, or New England Biolabs, unless otherwise specified, and digests were done according to the manufacturer's specifications.

The activity of β-galactosidase in yeast was assayed by use of a modification of the method of Guarente (1983). Four individual transformants were assayed after growth overnight in liquid SC lacking of the appropriate nutrients, utilizing 2% raffinose as a carbon source. The cells were pelleted by centrifugation, washed with water, and resuspended in Z buffer (100 mM NaPO₄ at pH 7.0, 10 mM KCl, 1 mM Mg(SO₄)₂, 38 mM β-mercaptoethanol). The OD₆₀₀ was determined for the washed cells, 40 µl of 0.1% SDS was added to 500 µl of cell suspension and mixed vigorously for 15 sec, followed by the addition of 60 µl of chloroform with repeated vortexing. ONPG (100 µl of a 4 mg/ml stock) was added, and the reaction was incubated at 30°C, until a yellow color began to appear, or for 15 min after which 0.5 ml of 1.0 M Na₂CO₃ was added to terminate the reaction. The reaction was centrifuged for 1 min in a microcentrifuge and the absorbance at 420 nm was determined. The activity of β-galactosidase in Miller units was calculated by use of the formula Units = A₄₂₀ × 1000 / (volume)(time)(OD₆₀₀) (Miller 1972).

Acknowledgments

We thank Drs. Barbara Triggs-Raine for critically reading this manuscript and Bernard A. Kunz for the yeast strain MKP^o. We also thank Ms. Sharon Rennie for expert technical assistance and Mr. Kevin C. Graham for verifying all deletion constructs in strains with Southern blot analysis. We also thank Dr. Daniel E. Gottschling for the generous gift of the yeast strains UCC1001 and UCC1083 and for sharing unpublished results. The work done by J.A.R. was performed in partial fulfillment of a undergraduate thesis for the University of Winnipeg. This work was supported by a studentship from the Manitoba Health Research Council to D.W.P. and a Manitoba Health Research Council Scholarship and a grant from the Medical Research Council of Canada (MT-11373) to R.D.G.

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Genes Dev. 1994, **8**:

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