The *Saccharomyces cerevisiae* DNA repair gene *RAD25* is required for transcription by RNA polymerase II

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The *RAD25* gene of *Saccharomyces cerevisiae* is required for excision repair of ultraviolet-damaged DNA and, in addition, is essential for viability. *RAD25* shares a high degree of homology with the human *ERCC3/XPBC*-encoded protein, and the yeast and human proteins resemble one another in containing the conserved ATPase/DNA helicase sequence motifs. To determine the nature of the essential role of *RAD25*, we have isolated a recessive temperature-sensitive conditional lethal mutation of the gene and have examined its effect on transcription. Upon shift to the nonpermissive temperature, the *rad25* temperature-sensitive (*ts*) mutant stops growth rapidly and shows a large decrease in the synthesis of poly(A)⁺ RNA. Transcription of a large number of yeast genes, including *HIS3*, *TRP3*, *STE2*, *MET19*, *RAD23*, *CDC9*, and *ACT1* is inhibited at the restrictive temperature in the *rad25 ts* mutant, and the galactose-inducible synthesis of *GAL7* and *GAL10* mRNAs is also severely affected by the loss of *RAD25* activity. These findings implicate a general requirement of *RAD25* in RNA polymerase II transcription.

[Key Words: *RAD25* gene; DNA repair; *S. cerevisiae*; RNA polymerase II; transcription; UV-damaged DNA]

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In eukaryotes, nucleotide excision repair of ultraviolet (UV)-damaged DNA is a complex process involving a large number of genes. In humans, xeroderma pigmentosum (XP), an autosomal recessive disorder, results from a deficiency in excision repair of DNA damage caused by UV light. XP patients exhibit extreme skin sensitivity to sunlight and pigmentation abnormalities, and suffer from a high incidence of skin cancers. Cell fusion studies have indicated the existence of seven excision-deficient XP complementation groups, XP-A to XP-G (Cleaver and Kraemer 1989; Vermeulen et al. 1991). In addition, several complementation groups have been identified among UV-sensitive excision repair-deficient rodent cell lines (Thompson et al. 1988; Hata et al. 1991; Stefanini et al. 1991). Many of the human excision repair genes have been cloned by complementing the excision repair deficiency of rodent cell lines, and these genes have been designated ERCC (excision repair cross complementing), whereas others have been cloned by direct complementation of the excision repair deficiency of XP cell lines (Park et al. 1992 and references therein).

In the yeast *Saccharomyces cerevisiae*, a total of 11 genes have been identified thus far to have a role in nucleotide excision repair. Mutations in six of these genes,

RAD1, *RAD2*, *RAD3*, *RAD4*, *RAD10*, and *RAD14*, cause extreme UV sensitivity and render cells defective in the incision step of excision repair (Reynolds and Friedberg 1981; Wilcox and Prakash 1981; Bankmann et al. 1992). Mutations in *RAD25(SSL2)* also confer increased UV sensitivity as a result of a deficiency in excision repair (Gulyas and Donahue 1992; Park et al. 1992). The protein products of these seven genes may be involved in damage recognition and in the endonucleolytic scission of the damaged DNA strand. Mutations in the remaining four genes, *RAD7*, *RAD16*, *RAD23*, and *MMS19*, cause a moderate increase in UV sensitivity, and these mutants exhibit a limited deficiency in excision repair (Wilcox and Prakash 1981; Miller et al. 1982a,b), suggesting a role for these genes in increasing the proficiency of excision repair. The excision repair genes have been conserved to a remarkable degree among eukaryotes, and a homolog has been identified in *S. cerevisiae* for each of the cloned human genes (Park et al. 1992 and references therein). The very high degree of conservation of excision repair genes among eukaryotes, from yeast to humans, provides strong support for the use of yeast as a model for understanding the role of these genes in humans.

Among the yeast excision repair genes, *RAD3* and *RAD25* are of particular interest, as these genes are essential for cell viability in addition to their role in excision repair (Higgins et al. 1983; Naumovski and Friedberg 1983; Gulyas and Donahue 1992; Park et al. 1992). Both genes also resemble one another in containing the

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conserved sequence motifs found associated with proteins that hydrolyze ATP or other nucleotides and possess a DNA or RNA helicase activity. These sequence motifs are also present in RAD16 (Bang et al. 1992; Manhaupt et al. 1992; Schild et al. 1992). RAD3-encoded protein contains a single-stranded DNA-dependent ATPase activity, and it also possesses DNA and DNA-RNA helicase activities (Sung et al. 1987a,b; Bailly et al. 1991). Mutation of the Lys-48 residue in the Walker type-A nucleotide-binding domain to arginine abolishes the RAD3 ATPase and helicase activities but not the ability to bind ATP. The *rad3 Arg48* mutation has no effect on cell viability but impairs the excision repair function of the protein (Sung et al. 1988).

RAD25 is a homolog of human *XPBC/ERCC3*-encoded protein, and the yeast and human proteins share 55% identical and 72% conserved amino acid residues (Gulyas and Donahue 1992; Park et al. 1992). The biochemical activity of RAD25- or *XPBC/ERCC3*-encoded proteins has not yet been identified; however, the presence of conserved helicase sequence motifs suggests that RAD25 and its human counterpart might possess ATPase/DNA helicase activities. A change of Lys-392 to arginine in the conserved Walker type-A nucleotide-binding sequence motif in RAD25 is lethal, suggesting an essential role of the putative ATPase/DNA helicase activity in viability (Park et al. 1992).

To define the nature of the essential role of RAD25 *in vivo*, we have generated a temperature-sensitive mutant allele of the gene and have determined the effect of this mutation under nonpermissive conditions on different biological processes, including transcription from a variety of promoters. *In vivo* studies such as those reported here are essential for assessing the role of the gene under normal physiological conditions. Interestingly, our studies indicate a requirement of RAD25 in RNA polymerase II-mediated transcription.

Results

Identification of a temperature-sensitive *rad25* mutant allele

A temperature-sensitive allele of RAD25 was isolated using the plasmid shuffle technique. In brief, a *TRP1* centromeric plasmid carrying the RAD25 gene was mutagenized by treatment with hydroxylamine *in vitro*, and the plasmid was introduced into the *rad25Δ* strain harboring the RAD25 gene on a *URA3* centromeric plasmid. The *URA3* plasmid was subsequently lost by growth of cells on plates containing 5-fluoro-orotic acid (5-FOA), and the phenotypic effects of the mutagenized plasmid on growth and UV response was determined.

In this manner, we identified a mutant allele, *rad25-ts₂₄*, that causes temperature-sensitive growth. Strains carrying this mutation grow normally at 25°C but fail to grow at 37°C (Fig. 1A). At the permissive temperature, the growth rates of the mutant and wild-type strains were nearly identical in liquid medium. At the restrictive temperature, the wild-type strain continues to grow,

Figure 1. Characterization of the *rad25-ts₂₄* mutation. (A) Growth of wild-type and *rad25-ts₂₄* strains on YPD plates at 25°C and 37°C. (Upper left) Strain LP3041-6D (*RAD*⁺); (upper right) strain EPY82-0 (*rad25Δ* strain carrying the RAD25 gene on *CEN* plasmid pEP39-0); (bottom) strain EPY82-24 (*rad25Δ* strain carrying the *rad25-ts₂₄* mutant gene on *CEN* plasmid pEP39-24). (B) Survival after UV irradiation of wild-type and *rad25* mutant strains. (●) Strain EPY82-0 (*RAD25*); (△) strain EPY82-24 (*rad25-ts₂₄*); (○) strain EPY88 (*rad25_{799am}*). After UV irradiation, plates were incubated at 25°C. Similar results were obtained with incubation of plates at 30°C. (C) Identification of the amino acid changes in the *rad25-ts₂₄* mutation. Codons 552–556 in the RAD25 and *rad25-ts₂₄* genes are shown.

whereas the mutant cells undergo between one to two cell divisions before ceasing growth (Fig. 2A). At 37°C, the mutant cells retain normal viability for many hours, as judged by examining the viability of cells upon shifting them back to the permissive temperature. At the

Figure 2. Growth of and macromolecular synthesis in EPY82-0 (*RAD25*) and EPY82-24 (*rad25-ts₂₄*) strains. (A) Growth curves of EPY82-0 (*RAD25*) and EPY82-24 (*rad25-ts₂₄*) in SC - Trp medium. Strains were grown at 25°C as described in Materials and methods. At time 0, the cultures were divided: Half of the culture was left at 25°C, the other half was shifted to 37°C, and the cell density was determined (OD_{600}) at the indicated time points. (B, C, and D). DNA, RNA, and protein synthesis, respectively. DNA, RNA, and protein synthesis were determined by continuous labeling as described in Materials and methods. [3H]Uracil for DNA and RNA synthesis or [^{35}S]labeled protein labeling mix for protein synthesis was added, and cultures incubated for 20 min at 25°C to allow for equilibration of pools. Cultures were then split (time 0). Half of the culture was left at 25°C, and the other half was incubated at 37°C. The counts incorporated during the first 20 min of equilibration at 25°C were subtracted from each sample obtained. EPY82-0 (*RAD25*) strain at 25°C (○) and at 37°C (●); EPY82-24 (*rad25-ts₂₄*) strain at 25°C (△) and at 37°C (▲).

restrictive temperature, the *rad25-ts₂₄* cells do not arrest at any particular cell division cycle stage.

The human XPB11BE frameshift mutation produces a protein altered in the 43 carboxy-terminal residues and causes a deficiency in excision repair (Weeda et al. 1990). The *rad25_{799am}* frameshift mutation in yeast changes amino acid residue 798 from valine to cysteine, and the encoded protein lacks the 45 carboxy-terminal residues. This mutation causes increased UV sensitivity but has no effect on viability or growth rate (Park et al. 1992). To determine whether the *rad25-ts₂₄* mutation affected the DNA repair role of the gene, we examined the UV sensitivity of the *rad25-ts₂₄* mutant at 25°C and 30°C. The UV survival of the *rad25-ts₂₄* mutant was identical to that of the wild-type strain (Fig. 1B).

By subcloning the various DNA fragments from the *rad25-ts₂₄* gene into the *RAD25* gene, the temperature-sensitive mutation was mapped to the 1-kb *ApaI-SphI* fragment that lies in the open reading frame of the gene. This fragment carries two GC → AT transitions, resulting in the conservative substitution of valine to isoleucine at amino acid position 552, and in the substitution of glutamic acid to lysine at position 556 (Fig. 1C). This region of the protein lies between the conserved helicase sequence motifs III and IV, and both the valine and glutamic acid residues are conserved at the corresponding positions in *RAD25* and its human homolog XPBC/ERCC3 (Gulyas and Donahue 1992; Park et al. 1992).

Effects of the *rad25 ts* mutation on macromolecular synthesis

The wild-type and *rad25 ts* strains were grown to early log phase at the permissive temperature, [3H]uracil was added to examine DNA and RNA synthesis, and [^{35}S]labeled protein-labeling mix (New England Nuclear) was added to examine protein synthesis. Twenty minutes after the addition of radioactive label, the cultures were divided and incubated at 25° and 37°C, and incorporation of label into macromolecules was determined. The DNA, RNA, and protein synthesis occurred at about the same rate in the wild-type and the mutant strain at the permissive temperature (Fig. 2). In contrast, in the *rad25* temperature-sensitive (*ts*) mutant, RNA synthesis was inhibited almost immediately upon transfer to the restrictive temperature and only minimal incorporation of radioactive precursor occurred subsequently (Fig. 2C, right). DNA synthesis occurred normally in the mutant ≤0.5 hr after shift to the restrictive temperature but fell at later time periods (Fig. 2B, right). The effect of the temperature-sensitive mutation on protein synthesis was less severe. In the mutant, protein synthesis continued at the normal rate for 1 hr at the restrictive temperature. Afterward, the rate of protein synthesis in the mutant was lower at 37°C than in the culture kept at 25°C. However, a slow and gradual incorporation of radioactive precursors into proteins continued to occur in the mutant throughout the 4-hr incubation period at 37°C (Fig. 2D, right). Two additional experiments gave results similar to those shown in Figure 2.

The simultaneous defects in DNA and RNA synthesis in the *rad25 ts* mutant are unlikely to arise from a primary defect in DNA synthesis, as the *S. cerevisiae* temperature-sensitive cell division cycle mutants *cdc7*, *cdc21*, and *cdc28*, which are defective in DNA replication, incorporate radioactive precursors into RNA at the same rate at the restrictive and the permissive temperatures (Hartwell 1973). Unlike the mutants defective in DNA synthesis that arrest cell division at the restrictive temperature at a discrete stage (Culotti and Hartwell 1971), the *rad25 ts* mutant resembles the mutants defective in RNA synthesis, which exhibit an asynchronous distribution of different cell cycle stages upon arrest at the restrictive temperature (Nonet et al. 1987; Cormack and Struhl 1992).

To check whether the RNA synthesis defect might be the primary cause of the DNA and protein synthesis defects observed in the *rad25 ts* mutant, we examined the *S. cerevisiae rpb1-1* mutation to determine whether it produces the same effects on macromolecular synthesis as the *rad25 ts* mutation. The *RPB1* gene encodes the largest subunit of RNA polymerase II. At the restrictive temperature, mRNA synthesis drops rapidly in the *rpb1-1* mutant; and as a consequence of mRNA depletion, rRNA synthesis also declines in the mutant (Nonet et al. 1987). As shown in Figure 3, in the *rpb1-1* mutant at the restrictive temperature, incorporation of radioactive precursors into RNA and DNA halted rapidly; however, protein synthesis continued but at a rate much lower than that observed at the permissive temperature. Thus, the effects of the *rpb1-1* allele on macromolecular synthesis are similar to that of the *rad25 ts* mutation. The *rpb1-1* mutant further resembles the *rad25-ts₂₄* mutant in the lack of cell cycle arrest at a discrete stage at the nonpermissive temperature (Nonet et al. 1987).

Inhibition of poly(A)⁺ RNA synthesis in the *rad25 ts* mutant

To characterize further the defect in RNA synthesis in the *rad25 ts* mutant, we measured the rate of total cellular RNA synthesis by pulse-labeling cells with [³H]uracil after a shift to the restrictive temperature. As shown in Figure 4A, a transient drop in the rate of RNA synthesis characteristic of the heat shock response occurs in the wild-type strain (Himmelfarb et al. 1987). Full recovery from heat shock occurs in ~45 min, after which wild-type cells continue RNA synthesis at the normal rate. In the *rad25 ts* strain, the rate of RNA synthesis drops to ~10% of the initial rate and remains at this low level at the later time periods (Fig. 4A).

To determine whether transcription by RNA polymerase II was affected in the *rad25-ts₂₄* mutant, poly(A)⁺ RNA was purified from total RNA pulse-labeled with [³H]uracil. In the wild-type strain, after the initial heat shock response at 37°C, a normal rate of poly(A)⁺ RNA synthesis resumed at 45 min (Fig. 4B). In the *rad25 ts* strain, however, the rate of poly(A)⁺ RNA synthesis fell dramatically upon shift to the restrictive temperature

Figure 3. Macromolecular synthesis in the *ts rpb1-1* mutant strain Y260 at 25°C and 37°C. DNA, RNA, and protein synthesis were determined by continuous labeling as described in Materials and methods and in the legend to Fig. 2. (Δ) 25°C; (▲) 37°C.

and stayed at ~10% the rate seen in the wild-type strain (Fig. 4B).

Defective mRNA synthesis in the *rad25 ts* mutant

The effects of the *rad25 ts* mutation on poly(A)⁺ RNA synthesis indicated an involvement of *RAD25* in RNA polymerase II transcription. To examine further the role of *RAD25* in polymerase II transcription, we determined, by Northern blot analysis, the steady-state levels of mRNAs encoded by the *HIS3*, *TRP3*, *STE2*, *MET19*, *RAD23*, *CDC9*, and *ACT1* genes in the wild-type and *rad25 ts*

Figure 4. Rates of total cellular RNA and poly(A)⁺ RNA synthesis in EPY82-0 (*RAD25*) and EPY82-24 (*rad25-ts₂₄*) strains. (A) Rates of total RNA synthesis. Rates of total RNA synthesis were determined by pulse-labeling as described in Materials and methods. Total cellular RNA was isolated from each [³H]uracil pulse-labeled cell sample. Radioactivity in the same amount of total RNA (2 μg) of each sample was determined by liquid scintillation counting. For the samples pulse labeled at 37°C, the incorporation of [³H]uracil (cpm/μg of total RNA) was normalized by the counts present in the sample that was pulse labeled at 25°C (time 0). (B) Rates of poly(A)⁺ RNA synthesis. poly(A)⁺ RNA was isolated from the same amount of [³H]uracil pulse-labeled total RNA (100 μg) by PolyATract mRNA purification system (Promega). Radioactivity in isolated poly(A)⁺ RNA was determined by liquid scintillation counting. For the samples pulse-labeled at 37°C, incorporation of [³H]uracil was normalized by the counts in the sample that were pulse-labeled at 25°C (time 0). (●) EPY82-0 (*RAD25*); (▲) EPY82-24 (*rad25-ts₂₄*).

strain before [0-min sample] and after transfer of cultures to 37°C (Fig. 5). The steady-state levels of mRNAs are a function of transcription rates and degradation rates. Upon transfer to the nonpermissive temperature, a defect in mRNA synthesis in the *rad25 ts* mutant would result in a reduction in the steady-state level of preexisting mRNAs. In the absence of new mRNA synthesis, the rate of reduction would be a function of mRNA decay rate. In the RNA polymerase II *rpb1-1* mutant and in

the TATA-binding protein *TBP ts* mutant, steady-state levels of mRNAs decrease upon shift of cells to the nonpermissive temperature (Nonet et al. 1987; Cormack and Struhl 1992). In our studies we included the *HIS3*, *TRP3*, and *STE2* genes because the half-lives of their encoded mRNAs are very short ($t_{1/2} < 7$ min) (Herrick et al. 1990; Cormack and Struhl 1992). Therefore, mRNAs synthesized before the temperature shift should not interfere significantly with the analyses. In wild-type cells, a shift to 37°C resulted in a transient reduction in *HIS3* and *TRP3* mRNA levels as a result of heat shock, followed by recovery to near normal levels at the subsequent time points. In contrast, in the *rad25 ts* strain, during the entire 3-hr incubation period at 37°C, the levels of *HIS3* and *TRP3* mRNAs remained at only ~10% of the level seen in the mutant strain before the temperature shift. The *rad25 ts* mutation also caused a large reduction in the transcription of the *STE2* gene.

Of the four other genes examined, transcription of *MET19* is very sensitive to the loss of *RAD25* activity. In the wild-type strain, *MET19* mRNA levels showed no heat shock response and remained nearly constant at 37°C, whereas in the *rad25 ts* strain, a rapid decline occurred in the level of *MET19* mRNA, and for all time points at 37°C, mRNA levels in the mutant strain were ~10-fold lower than the level before the temperature shift. The *rad25 ts* mutation also caused a reduction in the transcription of the DNA repair gene *RAD23*. In contrast to the nearly constant levels of *RAD23* mRNA at 37°C in the wild-type strain, a reduction of severalfold

Figure 5. Effect of the *rad25-ts₂₄* mutation on transcription of yeast genes. Shown are mRNA levels of *HIS3*, *TRP3*, *STE2*, *MET19*, *RAD23*, *CDC9*, and *ACT1* genes in the EPY82-0 (*RAD25*) and EPY82-24 (*rad25-ts₂₄*) strains. Cultures were grown at 25°C in SC - Trp medium, and transferred to 37°C, and the mRNA levels were examined at the indicated times by Northern hybridization.

occurred in the level of this mRNA in the mutant strain. The mRNA levels of the DNA ligase gene *CDC9* were also affected by the *rad25 ts* mutation. We also measured the levels of *ACT1* mRNA ($t_{1/2} > 25$ min) (Herrick et al. 1990) in the wild-type and *rad25 ts* strains. As observed for *MET19* and *RAD23*, the levels of *ACT1* mRNA in the wild-type strain showed no heat shock response and mRNA levels remained constant throughout the 3-hr incubation period at 37°C. The *rad25 ts* mutant strain, however, displayed a gradual decrease in *ACT1* mRNA levels, which fell to 10% of the normal level at 3 hr.

We also investigated the ability of *rad25 ts* mutant cells to transcribe the inducible genes *GAL7* and *GAL10* (Fig. 6). To examine the induction of these mRNAs, galactose was added and cultures were transferred immediately to 37°C. In the wild-type strain, *GAL7* mRNA appeared at 15 min and reached the maximal level at 1 hr after transfer to 37°C. The induction of *GAL10* mRNA occurs more slowly. The *rad25 ts* strain exhibited greatly reduced synthesis of both mRNAs. Thus, our studies indicate that the *rad25 ts* mutant is defective in both basal and activated transcription-dependent on RNA polymerase II.

RNA polymerase III transcription in the *rad25 ts* mutant

To determine whether the *rad25 ts* mutation affects genes transcribed by RNA polymerase III, we examined the transcription of tryptophan and isoleucine tRNA genes. To avoid the problem associated with the high stability of tRNAs, we used hybridization probes that are complementary to the intron sequences present in these tRNAs. Because introns are processed very rapidly, with a half-life of <3 min (Knapp et al. 1978; Cormack and Struhl 1992), the levels of precursor tRNAs containing the intron sequences should reflect the rate of transcription of these genes. As shown in Figure 7, transcription of either tryptophan or isoleucine tRNA was not affected by the *rad25 ts* mutation.

Effect of the *rad25 ts* mutation on rRNA synthesis

Results with incorporation of [³H]uracil into total RNA (see Fig. 2C) strongly suggested that the synthesis of

Figure 6. Galactose-inducible synthesis of yeast *GAL7* and *GAL10* mRNAs is inhibited in the *rad25-ts₂₄* mutant strain. The EPY82-0 (*RAD25*) and EPY82-24 (*rad25-ts₂₄*) strains were grown in YP5% glycerol medium. Galactose was added to 2%, and cultures were shifted immediately to 37°C. mRNA levels were examined by Northern blot analyses at the indicated time periods after shifting the cultures to 37°C.

Figure 7. Levels of intron-containing tRNA precursors of the isoleucine (tRNA^I) and tryptophan (tRNA^W) tRNA genes in the EPY82-0 (*RAD25*) and EPY82-24 (*rad25-ts₂₄*) strains after a shift to 37°C.

large rRNAs is affected in the *rad25 ts* mutant, because most RNA synthesis is of this type under continuous labeling conditions (Mann et al. 1992). The synthesis of RNA polymerase I-dependent rRNAs was examined directly by subjecting total RNA pulse-labeled with [³H]uracil to gel electrophoresis and fluorography. Upon transfer to 37°C, wild-type cells display a transient decrease in the rate of synthesis of all polymerase I-dependent rRNA species (35S, 27S, 25S, 20S, and 18S), followed by recovery to normal levels by ~45 min (Fig. 8). In contrast, in the *rad25 ts* cells, rRNA levels decreased with temperature shift and remained low throughout the 3-hr incubation period (Fig. 8). The decrease in rRNA synthesis in the *rad25 ts* mutant could be the result of shutdown of mRNA synthesis in the mutant. Loss of *RPB1* function also causes a rapid decline in rRNA synthesis; this has been suggested to be a consequence of the stringent response that shuts off rRNA synthesis under conditions when gene expression is affected (Nonet et al. 1987).

Discussion

Requirement of *RAD25* in RNA polymerase II transcription

In this paper we report our studies with a *rad25-ts₂₄* allele to determine the role of *RAD25* in vivo. A rapid

Figure 8. Levels of rRNAs transcribed by RNA polymerase I in the EPY82-0 (*RAD25*) and EPY82-24 (*rad25-ts₂₄*) strains. Cells were pulse labeled with [³H]uracil for 10 min at 25°C (0-min sample). Aliquots were transferred to 37°C and pulse labeled for 10 min, 15 min, 30 min, 45 min, 1 hr, 2 hr, and 3 hr after shift to 37°C. Total cellular RNA was isolated and separated on an agarose gel and fluorographed.

decline in RNA synthesis occurs in the *rad25 ts* mutant upon shift to the nonpermissive temperature. DNA synthesis also ceases in the mutant soon after the temperature shift, whereas the rate of protein synthesis declines gradually after the temperature shift. In all of these respects, the *rad25 ts* mutation resembles the *rpb1-1* allele of the gene encoding the largest subunit of RNA polymerase II.

The most interesting finding to emerge from this study is that *RAD25* is required for transcription by RNA polymerase II. A deficiency in *RAD25* activity results in a rapid decline in the rate of poly(A)⁺ RNA synthesis, and our findings indicate that *RAD25* is required for transcription of a large number of yeast genes—*HIS3*, *TRP3*, *STE2*, *MET19*, *RAD23*, *CDC9*, and *ACT1*. The galactose-inducible synthesis of *GAL7* and *GAL10* mRNAs is also affected severely by the loss of *RAD25* activity. Thus, our studies indicate a general requirement of *RAD25* in transcription by RNA polymerase II.

The shift of the *rad25 ts* mutant to the nonpermissive temperature does not affect the levels of intron-containing precursor RNAs encoded by two different tRNA genes, suggesting that *RAD25* does not affect RNA polymerase III-dependent transcription. RNA polymerase I-dependent rRNA synthesis, however, is hampered in the *rad25 ts* mutant. In this respect, the *rad25 ts* mutation resembles the *rpb1-1* mutation, which also causes a reduction in rRNA synthesis at the nonpermissive temperature, and which has been suggested to be a consequence of the stringent response (Nonet et al. 1987). Deprivation of amino acids in yeast results in the stringent response and causes a decrease in the rate of synthesis of rRNA and ribosomal proteins (Woelford and Warner 1991). The cessation of mRNA synthesis and the accompanying reduction in protein synthesis upon shift of the *rpb1-1* or the *rad25-ts₂₄* mutant to the nonpermissive temperature produces conditions similar to those that produce the stringent response. As suggested previously for the effects of *rpb1-1* on rRNA synthesis (Nonet et al. 1987), the reduction in the rate of rRNA synthesis in the *rad25-ts₂₄* mutant may be attributable to the stringent response rather than to a direct involvement of *RAD25* in RNA polymerase I transcription; however, that possibility cannot be ruled out entirely at this point.

The gradual decrease in protein synthesis in the *rad25 ts* mutant resembles that observed in the *rpb1-1* mutant and is likely a reflection of arrested mRNA synthesis. The nearly identical effect of the *rad25 ts* allele on DNA synthesis as that of the *rpb1-1* allele is also likely to be a consequence of cessation of mRNA synthesis.

Possible role of *RAD25* in transcription initiation

Promoter-specific initiation of transcription by RNA polymerase II requires the binding of the transcription factor TFIID, which recognizes the TATA box in the promoter, followed by the assembly of transcription factors TFIIA, TFIIB, TFIIIE, TFIIF, TFIIJ, and TFIIF, at the promoter site into an inactive preinitiation complex (Bu-

ratowski et al. 1989; Zawel and Reinberg 1993). Conversion of this preinitiation complex to an active initiation complex capable of initiating transcription requires the hydrolysis of the β - γ bond of ATP (dATP) (Bunick et al. 1982; Sawadogo and Roeder 1984). The available evidence suggests that in addition to the phosphorylation of the carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II, ATP hydrolysis serves an additional role, because RNA polymerase II lacking CTD is capable of initiating transcription from some promoters in vitro (Zawel and Reinberg 1993), and accurate initiation by RNA polymerase II missing this domain still requires ATP (Laybourn and Dahmus 1990). In this other role, ATP hydrolysis is used in the unwinding of the duplex DNA at the site of transcription initiation, facilitating the formation of an open complex (Wang et al. 1992). A DNA-dependent ATPase (dATPase) activity is found in transcription factor b from yeast (Feaver et al. 1991), factor δ from rat (Conaway and Conaway 1989), and factor TFIIF in humans (Schaeffer et al. 1993). These closely related multisubunit factors also contain a protein kinase activity that phosphorylates the CTD of RNA polymerase II (Feaver et al. 1991; Lu et al. 1992; Serizawa et al. 1992). Recently, it has been shown that a DNA helicase activity is associated with transcription factor TFIIF (Schaeffer et al. 1993). Furthermore, the *ERCC3/XPBC* gene product copurifies with this factor and comprises the largest (89 kD) subunit of TFIIF (Schaeffer et al. 1993). The presence of helicase motifs in the *ERCC3/XPBC*-encoded protein is consistent with the possibility that the observed DNA helicase activity in TFIIF derives from this protein. Because *RAD25* shares a high degree of homology (55% identical and 72% conserved residues) with the *ERCC3/XPBC*-encoded product, our findings predict a similar in vivo requirement of the human gene in transcription by RNA polymerase II. *RAD25* is essential for cell viability, and the presumed ATPase/helicase activity in *RAD25* serves an essential role, because mutation of the Lys-392 residue to arginine in the conserved Walker type-A nucleotide-binding motif is lethal (Park et al. 1992). These observations suggest that DNA unwinding during transcription initiation constitutes the essential role of *RAD25* and of its human counterpart *ERCC3/XPBC*.

RAD25/XPBC, Cockayne's syndrome, preferential repair of transcriptionally active genes, and other considerations

XP can occur alone or in association with Cockayne's syndrome (CS). The three known patients belonging to XP group B, in addition to suffering from XP, exhibit clinical features of CS. CS can occur without the accompaniment of XP, and two complementation groups, CS-A and CS-B, have been identified in cells from patients who show no manifestation of XP (Tanaka et al. 1981; Lehmann 1982). Patients suffering from XP alone exhibit high sensitivity to sunlight, pigmentation abnormalities, and a high incidence of skin cancers. Cells from XP pa-

tients show a high level of UV sensitivity and a deficiency in nucleotide excision repair. CS patients manifest severe dwarfism, mental retardation, progressive neurological degeneration, and cachexia (wasted appearance). These patients are sensitive to sunlight, but the pigmentation abnormalities and skin cancers found associated with XP are not seen in CS (Lehmann 1987; Nance and Berry 1992).

In both prokaryotes and eukaryotes, pyrimidine dimers are removed more efficiently from the transcribed strand than from the nontranscribed strand (Mellon et al. 1987; Mellon and Hanawalt 1989; Leadon and Lawrence 1992; Sweder and Hanawalt 1992). Cells from CS-A patients are defective in the preferential repair of the transcribed strand (Venema 1991). It appears unlikely, however, that the clinical manifestations of CS are the result of this defect, because even though XP cells belonging to, for example, complementation group A, are deficient in the repair of overall genome, (i.e., repair of both transcribed and nontranscribed strands), XP-A patients do not suffer from clinical symptoms of CS. We suggest that the association of CS and XP syndromes in XP group B patients arises from a defect in the capacity of the *ERCC3/XPBC*-encoded protein to function in nucleotide excision repair, resulting in XP symptoms, and a simultaneous reduction in the capacity of the protein to function in concert with the RNA polymerase II transcriptional machinery, resulting in CS. Thus, we suggest a deficiency in the transcription process itself rather than a defect in preferential repair of the transcribed strand as the cause of CS.

haywire encodes the *Drosophila* homolog of *RAD25*. Many of the *haywire* mutant alleles are recessive lethal. Some viable *haywire* alleles cause UV sensitivity, neurological abnormalities, and sterility (Mounkes et al. 1992). The *haywire* gene was identified initially by a recessive *hay^{nc2}* mutation that failed to complement certain recessive mutations in the *B2t* gene that encodes a testes-specific β -tubulin isoform. *hay^{nc2}* homozygous flies are male sterile and suffer from defects in testes-specific β -tubulin-dependent processes during spermatogenesis. The fertility of homozygous *hay^{nc2}* females is greatly reduced and *hay^{nc2}* is a temperature-sensitive semilethal mutation (Regan and Fuller 1988). A deficiency in transcription could account for the various phenotypes associated with the *hay^{nc2}* mutation.

The *RAD25* gene was isolated in one study as a suppressor of the *His4⁻* phenotype of cells carrying an artificial stem-loop structure in the 5'-untranslated region of the *HIS4* transcript and hence named *SSL2* (suppressor of *stem loop*). On the basis of this suppression effect, it has been proposed that *RAD25(SSL2)* functions in the translation initiation process by unwinding the stem-loop structure, thereby allowing for the translation of *HIS4* mRNA (Gulyas and Donahue 1992). In view of our findings, these results can now be explained by an effect of the *SSL2-1* mutation on transcription. Enhancement of the transcription of genes involved in the translation initiation process could produce the observed results.

The DNA repair and viability roles of RAD25 are mutationally separable

RAD25 alleles encoding the protein lacking the 45 or 94 amino acids from the carboxyl terminus confer a UV-sensitive phenotype but have no effect on cell viability or growth rate (Gulyas and Donahue 1992; Park et al. 1992). The *rad25-ts₂₄* allele, on the other hand, causes a conditional lethal phenotype but does not affect UV sensitivity. The ability to mutate one but not the other function of the gene suggests that *RAD25* contains separate domains for mediating its DNA repair and viability functions. The putative ATPase/DNA helicase activity in *RAD25*-encoded protein is likely to be essential for both functions, because the mutation of Lys-392 to arginine in the Walker type-A nucleotide-binding domain is lethal (Park et al. 1992) and because the *rad25 Arg392* mutant allele does not complement the UV sensitivity of the *rad25_{799am}* mutation that deletes the 45 carboxy-terminal amino acid residues of the protein. In transcription and in excision repair, the putative ATPase/DNA helicase activity of *RAD25* could be essential for unwinding DNA at the transcription initiation site and at the damage site. The possible existence of separate domains in *RAD25* suggests its involvement in complex formation with components of the transcriptional and excision repair machineries. *RAD25* could be targeted to the promoter site or to the damage site, depending on the proteins with which it is complexed, and thereby function in transcription or excision repair.

Materials and methods

Strains and plasmids

The strains EPY82-0, EPY82-24, and YC3-2 are derivatives of LP3041-6D, *MATa leu2-3 leu2-112 trp1-Δ ura3-52*, and carry a genomic deletion mutation of the *RAD25* gene (*rad25Δ*). Strain EPY82-0 carries the wild-type *RAD25* gene on the *TRP1* centromeric plasmid pEP39-0, whereas strain EPY82-24 carries the *rad25-ts₂₄* mutant gene on the *TRP1* centromeric plasmid pEP39-24. The *rad25Δ* strain with the *RAD25* gene carried on the *URA3* centromeric plasmid pEP23 is designated YC3-2.

The *rad25_{799am}* mutation changes codon 798 from GTT (Val) to TGT (Cys) and contains the nonsense codon TAG at position 799, thus producing a protein lacking the 45 carboxy-terminal amino acid residues, which has been described previously (Park et al. 1992). In strain EPY88, the genomic *RAD25* gene has been replaced by the *rad25_{799am}* mutant allele that results in UV sensitivity.

The strains DB1033, *MATa ura3-52*, and Y260, *MATa ura3-52 tpb1-1*, were kindly provided by Dr. R. Young (Massachusetts Institute of Technology, Cambridge).

YPD and synthetic complete medium lacking tryptophan (SC – Trp) were prepared as described (Sherman et al. 1986). YP5% glycerol medium consists of 1% yeast extract, 2% peptone, and 5% (vol/vol) glycerol.

Isolation of rad25 ts mutants

The *TRP1* plasmid pEP39-0 carrying the wild-type *RAD25* gene was treated with 10 M hydroxylamine (HA) at 75°C for 2.5 hr. Mutagenized pEP39-0 DNA was amplified in *Escherichia coli*

and then used to transform the yeast strain YC3-2 that has a genomic *rad25Δ* mutation and carries the wild-type *RAD25* gene on the *URA3* plasmid pEP23. Trp⁺ Ura⁺ transformants were tested for their ability to grow at the restrictive temperature of 37°C to eliminate any non-*rad25* or dominant *rad25* *ts* mutations. Transformants were then screened on media containing 5-FOA to select against the nonmutagenized *URA3* *RAD25* plasmid pEP23. The 5-FOA-resistant Trp⁺ Ura⁻ transformants were examined for temperature-sensitive growth and UV sensitivity. To verify that the observed phenotype was attributable to a mutation in the *RAD25* gene, the *HindIII*–*BamHI* DNA segment containing the entire *RAD25* gene was removed from a given mutagenized plasmid and inserted into the nonmutagenized *TRP1* centromeric vector, and the plasmid thus generated was used to replace the *RAD25* *URA3* plasmid pEP23 in the *rad25Δ* strain YC3-2 by plasmid shuffle. From a total of ~3000 Trp⁺ Ura⁻ transformants screened, we identified one plasmid-borne mutation that produced temperature-sensitive growth without concomitant UV sensitivity. This mutant allele was designated *rad25-ts₂₄*, and the yeast strain carrying the *rad25-ts₂₄* gene in plasmid pEP39-24 was designated EPY82-24.

Appropriate subcloning experiments indicated that the *rad25-ts₂₄* mutation was located in the 1-kb *ApaI*–*SphI* fragment in the open reading frame of the gene. The nucleotide change in the mutant allele was determined by DNA sequencing using the dideoxy chain termination method (Sanger et al. 1977) and deoxyadenosine 5'-[(α-³⁵S]thio]triphosphate (Biggin et al. 1983).

UV irradiation

Survival after UV irradiation was determined as described (Johnson et al. 1992). Briefly, cells were spread onto SC – Trp plates, exposed to UV irradiation, and incubated at 25°C, or at other temperatures, in the dark to avoid photoreactivation.

Determination of growth

Strains EPY82-0 (*RAD25*) and EPY82-24 (*rad25-ts₂₄*) were grown at 25°C in SC – Trp medium. When the density reached an OD₆₀₀ of ~0.3, the cultures were divided; half of the culture was placed at 25°C and the other half at 37°C. At the indicated times, the aliquots were removed and OD₆₀₀ was determined.

Macromolecular synthesis

In vivo synthesis of DNA and RNA in strains EPY82-0 (*RAD25*) and EPY82-24 (*rad25-ts₂₄*) was determined as described (Brill et al. 1987) with minor modifications. Briefly, cells were grown at 25°C in SC – Trp medium to an OD₆₀₀ of ~0.5. [5,6-³H]Uracil (Moravsek Biochemicals) was added to the cultures to a final concentration of 10 μCi/ml. The cultures were incubated at 25°C for 20 min for equilibration of the nucleotide pool. Half of the culture was then incubated at 25°C, and the other half was incubated at 37°C. To determine DNA synthesis, 0.2-ml aliquots were removed at various times and placed in 0.5 ml of stop solution (15% trichloroacetic acid (TCA), 200 μg/ml of thymine, and 50 mM sodium pyrophosphate) containing 0.1 ml of unlabeled stationary-phase yeast cells as carrier. The samples were pelleted, resuspended in 50 μl of 0.6 M NaOH, and left overnight at 37°C to allow for RNA hydrolysis. Samples were then neutralized with 6 M HCl and precipitated with 1 ml of stop solution on ice for 30 min. Precipitates were collected on Whatman GF-C filters, and the filters were washed, dried, and placed in Filter-count (Packard Instrument Inc.). Radioactivity was determined in a liquid scintillation counter. To determine RNA synthesis, the aliquots were removed at the indicated

times, placed in 2 ml of stop solution, and kept on ice for 30 min. Samples were filtered, washed, and dried, and radioactivity was determined as above.

³⁵S Labeled Protein Labeling Mix (New England Nuclear) was used for in vivo protein labeling at a final concentration of 10 μCi/ml. Aliquots (0.1 ml) were removed and placed in 3 ml of cold 5% TCA. The samples were then boiled at 100°C for 15 min and placed on ice for 20 min. The precipitates were filtered, washed, and dried, and radioactivity was determined as above.

Macromolecular synthesis was also examined in the wild-type strain DB1033 and the *rpb1-1* mutant strain Y260 (Nonet et al. 1987), using the methods described above.

Pulse-labeling of total RNA in vivo

Strains EPY82-0 (*RAD25*) and EPY82-24 (*rad25-ts₂₄*) were grown at 25°C in SC – Trp medium to an OD₆₀₀ of ~0.6 and divided into several aliquots. [5,6-³H]Uracil was added to one aliquot, and cells were pulse labeled at 25°C for 10 min (time 0 sample). The other aliquots were transferred to flasks prewarmed at 37°C and placed at 37°C. The 10-min pulse-labeling was performed at 37°C at 15, 30, 45, 60, 120, and 180 min after the shift to 37°C. The final concentration of [5,6-³H]Uracil used for pulse-labeling was 30 μCi/ml. After pulse labeling, the cultures were quickly chilled by pouring over frozen, crushed 1 M sorbitol. Cells were subsequently pelleted, quickly frozen in crushed dry ice, and used for preparing total ³H-labeled RNA as described below in Northern blot analysis.

Measurement of poly(A)⁺ RNA synthesis

The ³H-labeled poly(A)⁺ RNA was purified from the same amount of total cellular RNA isolated from cells pulse-labeled with [5,6-³H]Uracil as described above by using the PolyATtract mRNA purification system (Promega). Radioactivity in each sample was determined by liquid scintillation counting.

Fluorographic detection of de novo synthesis of rRNA

Total RNA (20 μg) obtained from cells pulse-labeled with [5,6-³H]Uracil was subjected to electrophoresis on a 3% NuSieve 3 : 1 agarose gel. The gel was treated with Amplify (Amersham), dried, and fluorographed at –70°C with an intensifying screen.

Northern blot analysis

Strains EPY82-0 (*RAD25*) and EPY82-24 (*rad25-ts₂₄*) were grown at 25°C to an OD₆₀₀ of ~0.6 in SC – Trp medium. A sample was removed from both cultures (0-min sample), and cultures were transferred immediately to 37°C in a water bath shaker. Samples were taken at 15, 30, 45, 60, 120, and 180 min after the temperature shift. Total cellular RNA was prepared as described (Madura and Prakash 1986). Briefly, cell pellets were resuspended in 350 μl of yeast lysis buffer [200 mM Tris-HCl (pH 7.5), 500 mM NaCl, 10 mM EDTA, 0.5% SDS, 10 mM iodoacetic acid, and 10 mM vanadyl ribonucleoside complex (BRL)] and broken by glass beads with 1 volume of phenol/chloroform. RNA was precipitated by ethanol, purified by RNase-free DNase I (Boehringer) treatment, and quantitated by spectrophotometry. An equal amount of RNA from each sample was denatured by glyoxal, fractionated on a 1.4% agarose gel, and electroblotted onto GeneScreen membrane (DuPont, New England Nuclear). Hybridization and washing were performed as described in the instruction manual. The DNA fragments used as hybridization probes for detecting various mRNAs were ³²P-labeled by the multiprimer DNA-labeling system (Amersham).

To examine the transcription of tRNA genes, RNA samples

were fractionated in a 3% NuSieve 3 : 1 agarose gel (FMC), and fractionated RNA was electroblotted onto GeneScreen (Du Pont, New England Nuclear). The oligonucleotides used for hybridization to intron-containing tRNA precursors were as follows, and they included only the internal portions of these introns: tRNA^I, AAAGGCCTGTTTGAAGGTCTTTGGCAC-AGAACTTCGGAAACCG (Ogden et al. 1984); tRNA^W, GC-AATCTTATTCGCGGAATTTCCAAGATTTA (Van Arsdell et al. 1987). The temperature for hybridization and washing was reduced appropriately according to the melting temperature of the oligonucleotide used.

The hybridization probes were used in excess, as verified by decreasing the amount of probe by over fivefold.

Transcription of all the genes was examined in at least two independent experiments, which gave similar results.

GAL genes induction

Strains EPY82-0 (RAD25) and EPY82-24 (*rad25-ts₂₄*) were grown at 25°C in YP5% glycerol medium to an OD₆₀₀ of ~0.5. Galactose was added to the cultures to a final concentration of 2%. The cultures were transferred immediately to the 37°C prewarmed flasks and placed at 37°C. Samples were removed at 0, 15, 30, 45, 60, 120, and 180 min after adding galactose and shifting the culture to 37°C and poured immediately over frozen crushed 1 M sorbitol. Cells were pelleted and quickly frozen in crushed dry ice. Total RNA was isolated from these cell samples and analyzed by Northern hybridization as described above.

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